



Report

**ASSESSMENT OF TOXIC
AND ECOTOXIC
PROPERTIES OF
NOVEL PROTEINS IN GMOS**

**Forschungsberichte der
Sektion IV**

Band 1 / 2008

Teil 1



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Part A: Assessment of toxic and anti-nutritive properties for humans

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Acronyms

ADI	Acceptable Daily Intake
AMFEP	Association of Manufacturers and Formulators of Enzyme Products, EU
AMP	Anti-microbial Peptides
BLAST	Basic Local Alignment Search Tool
Bt	Bacillus thuringiensis
CFR	Code of Federal Regulations
CFT	Channel Forming Toxins
CFU	Colony Forming Unit
DC	Decanoylcarnitine
EFSA	European Food Safety Authority
EP	End-Use Product
EPA	Environmental Protection Agency, USA
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration, USA
FDC	Food, Drug and Cosmetic
GEMS	Global Environment Monitoring System
GFP	Green Fluorescent Protein
GI	Gastro Intestinal
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
GMP	Genetically Modified Plant
GRAS	Generally Recognised as Safe
HSP	High-Scoring Pairs
IS	Immune System
IVD	In-Vitro Digestibility
JECFA	Joint FAO/WHO Expert Committee on Food Additives
OECD	Organisation for Economic Co-operation and Development
MP	Manufacturing-Use Product

MoS	Margin of Safety
PDB	Protein Data Bank
PFT	Pore Forming Toxins
PHA	Phytohemagglutinin
PI	Proteinase Inhibitors
PIP	Plant Incorporated Protectant
REBECA	Regulation of Biological Control Agents
RIP	Ribosome Inactivating Component
SCP	Scientific Committee on Plants
SGF	Simulated Gastric Fluids
SIF	Simulated Intestinal Fluids
SSC	Scientific Steering Committee
TA	Tartaric Acid
TMDI	Theoretical Maximum Daily Intake
WHO	World Health Organization

Zusammenfassung

Untersuchungen zur Toxizität von Proteinen sind ein Aspekt der Risikoabschätzung gentechnisch veränderter Pflanzen (GVP). In Zukunft könnte diesem größere Bedeutung zukommen, wenn die Exposition von neuen Proteinen in gentechnisch veränderten Pflanzen, die Konzentration in der Pflanze oder der Grad an Neuartigkeit der Proteine zunehmen oder wenn Nahrungsmittelpflanzen für die Herstellung von Proteinen für pharmazeutische und industrielle Zwecke verwendet werden (Molecular Farming).

Ziel der vorliegenden Studie ist es,

- die derzeitige Praxis der Abschätzung von toxischen und ökotoxischen Eigenschaften von neu exprimierten Proteinen in gentechnisch veränderten Pflanzen zu analysieren,
- Schwachstellen zu identifizieren und
- Vorschläge für Verbesserungen zu machen.

Allergene Eigenschaften von Proteinen wurden dabei explizit nicht berücksichtigt. Zu diesem Zweck wurden die toxikologische und ökotoxikologische Praxis der Risikoabschätzung von GVP durch die Antragsteller, die relevanten Gesetze und Leitlinien sowie die publizierte Literatur analysiert. Ergebnisse eines ExpertInnenworkshops, bei dem für die Studie relevante Hypothesen und Ansichten diskutiert wurden, wurden ebenso berücksichtigt wie mündliche und schriftliche Kommentare von ExpertInnen aus Wissenschaft, Industrie und Behörden. Die Analyse fokussiert auf die Datenbasis zur Toxizität und Ökotoxizität von Proteinen im Allgemeinen sowie spezifisch in Bezug auf Proteine, die in GVP eingebracht werden, wie beispielsweise bakterielle Cry-Proteine. Jene Hypothesen, die der toxikologischen und ökotoxikologischen Risikoabschätzung von GVP zugrunde liegen, wurden ebenso analysiert wie die angewandten Methoden für deren Bewertung. Darüber hinaus werden auch andere regulatorische Bereiche wie Lebensmittelenzyme und Biopestizide vergleichend angesprochen. Zudem werden mögliche Verbesserungen der toxikologischen und ökotoxikologischen Risikoabschätzung im Zusammenhang mit GVP vorgeschlagen und diskutiert.

Teil A: Abschätzung von humantoxischen und anti-nutritiven Eigenschaften

Teil A zur Bewertung von möglichen humantoxischen Eigenschaften von Proteinen analysiert Leitlinien und die Abschätzungspraxis in Bezug auf Zugang, Grundannahmen, Methoden und Art der berücksichtigten Informationen auf Basis von Risikoabschätzungsdossiers, Evaluierungen dieser Dossiers und wissenschaftlicher Literatur. Vergleichend wird die Vorgangsweise bei der Proteintoxizitätsabschätzung von Lebensmittelenzymen und Biopestiziden untersucht. Den Hintergrund bildet eine Zusammenschau der wissenschaftlichen Literatur zu Proteinen, die bei oraler Aufnahme toxische oder anti-nutritive Eigenschaften haben können.

Die Toxizitätsabschätzung von neu exprimierten Proteinen im Kontext von GVP erfolgt fallspezifisch (case-by-case). In den meisten Leitlinien und in der Praxis erfolgt die Gefahrenerkennung und -charakterisierung auf Basis

- i. der Charakterisierung des Donororganismus;
- ii. der Klärung der physiologischen Rolle, des biochemischen Wirkmechanismus und der Spezifität des Proteins;
- iii. der vorhandenen Erfahrungen mit dem Protein in der menschlichen Ernährung bzw. der Exposition;
- iv. von Sequenzhomologievergleichen zu bekannten Toxinen;
- v. von In-vitro-Verdauungsstudien und
- vi. von akuten Toxizitätstests mit Nagern.

Bei diesem Ansatz wurden folgende Schwachstellen identifiziert:

- Die Erfahrungen mit den neu exprimierten Proteinen in der menschlichen Ernährung bzw. Exposition beziehen sich zumeist nicht auf wissenschaftliche Studien; mitunter werden diese auch nicht mit dem identen Protein, sondern mit ähnlichen bzw. homologen Proteinen gewonnen.
- Am Beispiel von GVP, die Cry-Toxine exprimieren, lässt sich zeigen, dass auch nach mehr als 12 Jahren nach der Erstzulassung solcher GVP immer noch offene Fragen bezüglich möglicher physiologischer Effekte in Menschen bestehen.
- Für Sequenzhomologievergleiche zu bekannten Toxinen gibt es – anders als bei derartigen Vergleichen zu bekannten Allergenen – keine Leitlinien. Manche der Sequenzvergleiche datieren mehr als zehn Jahre zurück. Eine Aktualisierung wird unter Umständen von Firmenseite auf freiwilliger Basis durchgeführt, aber zumindest mehrheitlich nicht durch Behörden überprüft. Dies erfolgt auch nicht bei der Verlängerung der Marktzulassung oder bei der Zulassung von anderen GVP, die das gleiche Protein exprimieren.
- Das Testprotein für In-vivo-Toxizitätstests stammt nahezu ausschließlich aus mikrobieller Herstellung, da eine Reinigung aus Pflanzen aufgrund zu geringer Expressionslevel als zu aufwendig angesehen wird. Zudem werden Äquivalenzuntersuchungen von pflanzlichen mikrobiellen Proteinen in unterschiedlicher Weise durchgeführt.
- In-vitro-Verdauungsstudien weisen eine beträchtliche Variabilität im experimentellen Design auf, was die Resultate und Schlussfolgerungen beeinflussen kann. Zudem stellt sich die Frage nach der Relevanz dieser Studien für die In-vivo-Situation, in der beispielsweise der pH-Wert des Magens deutlich höher sein kann und das exprimierte Protein in einer Lebensmittelmatrix vor proteolytischem Abbau geschützt sein kann.
- Toxizitätstests mit Tieren werden als nicht notwendig angesehen, wenn die oben beschriebenen Studien bzw. Informationen nahe legen, dass das Protein über keine toxischen Eigenschaften verfügt. Mögliche Anlassfälle für In-vivo-Studien sind:
 - i. problematische/r oder unbekannte/r Wirkmechanismus oder Spezifität,
 - ii. toxische Wirkungen auf Säugetiere,
 - iii. nicht dokumentierte Exposition von Mensch und Tier,
 - iv. falls die geschätzte Exposition negative Gesundheitsfolgen auslösen könnte,
 - v. Änderungen der Primärstruktur und
 - vi. falls das Protein in In-vitro-Verdauungsstudien nicht rasch abbaubar ist.Vorgeschlagen werden je nach Leitlinie akute (USA) oder 28-Tage-subchronische Toxizitätsstudien (EU). Subchronische 28-Tage-Studien sind bislang mit wenigen Ausnahmen nicht durchgeführt worden.
- Subchronische 90-Tage-Fütterungsstudien mit der ganzen gentechnisch veränderten Pflanze und Nagern können zusätzliche sicherheitsrelevante Informationen für das neu exprimierte Protein ergeben, abhängig unter anderem vom Studiendesign, den spezifischen Proteineigenschaften und der Pflanzenspezies.
- Diese starke Gewichtung von Ex-vivo-Studien zu Ungunsten von In-vivo-Toxizitätstests lässt sich im Vergleich zu den regulatorischen Kontexten von Lebensmittelenzymen und Biopestiziden argumentieren, da in diesen der Fokus

der Tierstudien eher auf Begleitstoffen und Kontaminanten aus der Fermentation liegt (Mycotoxine und bakterielle Toxine). Auf der anderen Seite wird dies als problematisch angesehen, da Anzeichen von subchronischen Effekten nicht notwendigerweise in akuten Studien entdeckt werden. Der derzeitige Wissensstand suggeriert zwar, dass toxische Proteine sehr häufig akute Wirkungen haben, gleichzeitig scheint es aber kaum Forschungsanstrengungen zur Exploration von möglichen toxischen Effekten jenseits von akuten Wirkungen zu geben. Eine Ausnahme hiervon sind beispielsweise Lektine, bei denen allerdings nicht in jedem Fall klar ist, welche Effekte als toxisch und welche als anti-nutritiv eingestuft werden. Zudem ist unklar, ob diese Grenzziehung überhaupt von Bedeutung ist, da auch anti-nutritive Effekte bei längerer Exposition zu deutlichen Gesundheitsbeeinträchtigungen führen können.

- Ebenso problematisch ist der völlige Entfall von In-vivo-Toxizitätstests entsprechend den EFSA-Leitlinien – im Wesentlichen aus zwei Gründen: Erstens ist eine ausschließliche Beurteilung der Toxizität eines neu exprimierten Proteins aufgrund von Ex-vivo-Untersuchungen aufgrund der identifizierten Schwachstellen fragwürdig. Zweitens liegen noch keine Erfahrungswerte vor, die eine Einschätzung zulassen, ob solche Eigenschaften durch die Ex-vivo-Studien erkannt werden würden. Von den mehr als 40 verschiedenen Proteinen, bei denen eine Toxizitätsabschätzung durchgeführt worden ist, wurde bislang kein Protein als problematisch identifiziert und es wurden keine (weiteren) Toxizitätstests verlangt.
- Falls eine quantitative Expositionsabschätzung vorliegt, erfolgt diese häufig auf Basis der Proteinkonzentration in der Pflanze, auf Basis von Datenbanken zu Nahrungsmittelaufnahmen und worst-case-Annahmen, wie z. B. kein Proteinabbau und hoher Proteinanteil in der menschlichen Ernährung. Eine formale Risikocharakterisierung wird weniger häufig durchgeführt. Teilweise werden dabei toxikologische Endpunkte wie z. B. der NOAEL (no observed adverse effect level) auf Basis akuter Toxizitätstests berechnet, was im Widerspruch zur normalen toxikologischen Praxis steht, in der von mindestens 90 Tage dauernden subchronischen Tests ausgegangen wird.

Aufgrund dieser Analyse wird Forschungsbedarf zu möglichen toxischen Wirkungen jenseits von akuten Effekten, zur Relevanz der In-vitro-Verdauungsstudien für die In-vivo-Situation sowie zur Absorption von Proteinen aus dem Verdauungstrakt gesehen. Bedarf an Leitlinien auf EU-Ebene ist für das Design von In-vitro-Verdauungsstudien, Sequenzhomologievergleiche und für die Berücksichtigung von Erfahrungen mit vorheriger menschlicher Exposition gegeben. Klarstellungen sind ferner erforderlich bezüglich der Unterscheidung toxischer und anti-nutritiver Eigenschaften und der Festlegung, welche Effekte klar außerhalb des Fokus der GVP-Risikoabschätzung liegen. Außerdem sollte geklärt werden, in welchen Fällen eine Produktion des Testproteins aus Pflanzen durchführbar ist.

Teil B: Abschätzung der Ökotoxizität

Teil B analysiert und diskutiert die derzeitige Praxis der ökotoxikologischen Risikoabschätzung von in GVP eingebrachten Proteinen sowie die der Risikoabschätzung zugrunde liegenden Annahmen und angewandten Methoden. Zudem werden Fragen der Protein-Ökotoxizität von Bakterien, Pilzen und Pflanzen im Allgemeinen und spezifisch in Bezug auf die für GVP relevanten, bakteriellen Cry-Proteine angesprochen. Anträge, die den Anbau von GVP in der Europäischen Union inkludieren, gesetzliche Vorgaben und Leitlinien sowie die publizierte Literatur bilden die Grundlage dieser Analyse.

Die derzeitige Praxis der ökotoxikologischen Risikoabschätzung von bakteriellen Cry-Proteinen geht von folgenden Annahmen bezüglich der Protein-Ökotoxizität aus:

- i. Proteine können nur akut wirken;
- ii. Proteine wirken hoch spezifisch gegen einen bestimmten Schädling;
- iii. Proteine werden schnell im Ökosystem abgebaut;
- iv. mehrere in eine gentechnisch veränderte Pflanze eingebrachte Proteine interagieren nicht.

Aufgrund dieser Annahmen stützt sich die ökotoxikologische Risikobewertung von GVP derzeit auf akute Kurzzeit-Toxizitätstests mit wenigen, ausgewählten Nichtzielorganismen sowie Kurzzeittests zum Proteinabbau im Boden. Systematische Expositionsanalysen und Risikocharakterisierungen für Nichtzielorganismen fehlen meist.

Folgende Schwächen der derzeitigen Praxis der ökotoxikologischen Risikoabschätzung von in GVP eingebrachten Proteinen können somit festgestellt und Handlungsempfehlungen abgeleitet werden:

- Das Fehlen systematischer Expositionsanalysen von Nichtzielorganismen lässt die Verfügbarkeit von Cry-Proteinen über unterschiedliche Expositionswege wie direkte oder indirekte Nahrungsaufnahme bzw. über Abbauprodukte in Wasser, Boden oder Sedimenten unberücksichtigt. Hinsichtlich der Durchführung von Expositionsanalysen und Risikocharakterisierungen – wie dies auch in anderen regulatorischen Bereichen, z. B. mikrobiellen Pflanzenschutzmitteln, der Fall ist – sind daher Verbesserungen notwendig.
- Die derzeit vorgelegten Studien zur Ökotoxizität und zum Proteinabbau von Cry-Proteinen in GVP sind hinsichtlich des Versuchsdesigns, der Methode, der Auswahl der Testorganismen und der Testdauer als unzureichend zu bewerten. Wesentliche Erkenntnisse zur Proteintoxizität, die über die akut toxischen Wirkungen und die Kurzzeitpersistenz hinausgehen, bleiben in den GVP-Anträgen unberücksichtigt. Daher sind klare Vorgaben auf EU-Ebene für die Antragsteller in Bezug auf die Auswahl der Testorganismen, die zu verwendende Methodik sowie die Testparameter notwendig.
- Unklarheiten bestehen hinsichtlich der Spezifität und biologischen Wirksamkeit der in den ökotoxikologischen Tests verwendeten Cry-Proteine als Testsubstanz. Daher sollten bei der Auswahl der Testsubstanz die realen Bedingungen besser abgebildet und ganze Pflanzen bzw. Pflanzenteile in die Tests aufgenommen werden. Damit können Unterschiede zwischen Proteinen, die in Pflanzen bzw. Mikroorganismen exprimiert werden, sowie die Zusammensetzung der Wirtspflanze berücksichtigt werden.
- In den GVP-Anträgen wird derzeit für ökotoxikologische Tests selten auf standardisierte Testprotokolle zurückgegriffen. Eine Verwendung standardisierter Protokolle (z. B. OECD) ist jedoch unumgänglich, um valide Testergebnisse zu erhalten. Zudem ist eine Adaptierung der vorhandenen Testprotokolle oder die Entwicklung neuer Protokolle für ökotoxikologische Tests, die den spezifischen Anforderungen von GVP gerecht werden, nötig.
- Mögliche Interaktionen mehrerer ökotoxischer Proteine, die in eine GVP eingebracht werden, dürfen nicht unberücksichtigt bleiben und müssen in ökotoxikologische Bewertungen inkludiert werden.

Forschungsbedarf besteht insbesondere für die folgenden Bereiche:

- Es bestehen Unsicherheiten bezüglich der Spezifität und biologischen Wirksamkeit von Cry-Proteinen gegenüber Ziel- und Nichtzielorganismen im Allgemeinen und speziell in Bezug auf Cry-Proteine, die in ökotoxikologischen Tests verwendet werden (exprimiert in GVP oder Bakterien). Der derzeitige Wissensstand zur Ökotoxizität von Cry-Proteinen ist daher dringend zu verbessern.

- Die derzeitige Datenbasis über das Schicksal und Verhalten von Cry-Proteinen in unterschiedlichen Umweltkompartimenten sowie in der Nahrungskette ist äußerst limitiert. Mit dem großflächigen Anbau von GVP ist das Vorhandensein dieser Proteine in den verschiedensten Habitaten und Organismen zu erwarten. Deshalb ist ein fundiertes Verständnis des Verhaltens und der Persistenz dieser Proteine wesentlich.

Executive Summary

The overall goal of this report is to review the currently applied approaches in toxicity and ecotoxicity assessment of novel proteins in the context of genetically modified plants and to identify and discuss possible weaknesses and limitations.

This was done by a review of the toxicity and ecotoxicity assessment practice by notifiers, relevant guidance documents and the published literature. The results from a workshop identifying and discussing evidence and views relevant to this report are included as well as written comments from external scientific experts and stakeholders from industry and authorities. The review focuses on the knowledge base of toxicity of proteins in general, and the specific knowledge base of proteins expressed in genetically modified plants, the evidence considered in toxicity and ecotoxicity assessment and the methodologies used in notifications of genetically modified plants. Additionally, the report includes evidence and experience in the toxicity and ecotoxicity assessment of proteins from other regulatory contexts such as food additives and biopesticides.

Recommendations for possible improvements of toxicity and ecotoxicity assessment in the context of GM crops are discussed.

Part A: Assessment of toxic and anti-nutritive properties for humans

Part A reviews protein toxicity assessment in the context of GM plants and food; the overall approach, the methods used and the type of evidence considered. The analysis draws on both guidance documents and toxicity assessment practice. The latter is documented in risk assessment dossiers submitted in the course of the regulatory procedure, in published summaries and evaluations of such dossiers, and in the scientific literature.

The toxicity assessment of novel proteins in the context of GM plants and food as outlined in most guidance documents follows a case-by-case approach. In practice, hazard identification and characterisation essentially includes information on the history of human exposure/consumption, information on the source organism, the mode of action and specificity, potential homology to known toxins, in-vitro digestibility studies and acute toxicity studies in rodents. Exposure assessment includes information on expression levels in the plant and a description of food processing including studies on temperature stability. A calculation of the dietary intake of the novel protein based on food consumption databases and expression levels is not always provided.

According to recent guidance documents, toxicity tests might be waived in case a **history of safe consumption** of the introduced protein exists. A history of safe consumption frequently refers to anecdotal evidence and often to generic categories such as EPSPS proteins or Cry proteins “in general”, or technical grade **Bt** products. The absence of negative health impacts is usually not documented by epidemiological studies, which are thus unlikely to consider possible long-term and/or less severe effects. Moreover, even the anecdotal evidence is frequently limited to a few sentences. Despite its key role the concept of history of safe use has not been defined and is lacking scientific precision.

In the **description of the source organism** precise taxonomic identification is a prerequisite to track reports of any toxic or pathogenic properties which could have a bearing on the function of the isolated gene. Experience with source organisms is, so far, limited to microbial sources many of which are claimed to have some kind of history of human exposure.

The **mode of action**, understood as the physiological role of the protein and its underlying molecular mechanisms, and the specificity can be investigated for its relevance to human physiology. The mode of action is perhaps most extensively investigated with Cry proteins. Still, more than a decade after market commercialisation questions on the biochemical activity of **Bt** toxins in humans are being raised.

Information on the type of **food processing** can be relevant as it will determine the likelihood of the presence of functionally intact novel proteins in the final food product. Food processing often includes heat treatments and shifts in pH which both could inactivate food proteins. This is also the main reason for providing information on **temperature stability** though it is not always clear how these studies relate to the most relevant routes of food processing.

Amino acid sequence comparisons can establish similarity/homology to known toxins, the absence of which is taken as a confirmation of safety. In contrast to homology searches for allergenic epitopes there is no detailed guidance available for conducting such comparisons. In more recent practice the same approach as employed in allergenicity assessment (more than 35 % similarity within 80 consecutive amino acids) has been used. Homology comparisons reviewed in the course of this study differ in algorithm, parameters and databases used. Fast methods seem to be preferred to more sensitive methods. Some sequence comparisons have been conducted more than a decade ago and are still being referred to in recent risk assessment dossiers despite the fact that the number of sequence entries of proteins suspected or known to have toxic properties have dramatically increased. According to company information homology searches are regularly updated but not submitted and also not always included in submissions for the renewal of market authorisations.

The **test protein** used in animal studies is normally purified from micro-organisms. This practice has been justified with the very low levels of protein expression in plant tissue. As plant and microbial proteins can differ with respect to amino acid sequence, folding and post-translational processing (e.g. glycosylation) structural and functional equivalence of test protein and plant derived protein has to be established. This adds additional uncertainties to the interpretation of animal toxicity tests. An estimate of the amount of test protein needed revealed that in case of expression levels beyond 100 µg/g fresh weight it might be feasible to purify sufficient amounts of the novel protein from the plant to conduct a 28-day subacute study with plant-derived protein. The technical feasibility and the costs arising from plant protein purification might, however, require more detailed study and discussion.

In-vitro digestibility (IVD) tests are considered to provide evidence on the fate of the protein in the human gastrointestinal (GI) tract following ingestion. A comparison of the experiments described in risk assessment dossiers and in the scientific literature revealed considerable variations in the protocols with the amount of pepsin per unit test protein being most striking. In the absence of more detailed guidance IVD studies are likely to lead to arbitrary results. Furthermore, the relevance of IVD for the in-vivo situation appears to be questionable. As opposed to single substance digestion in-vitro, in-vivo proteins can be protected in a food matrix; GI conditions can differ depending on the type of food, on age, stomach content, existing pathologies and medication. Contradicting evidence is so far available from animal studies confirming both rapid and lower rates of digestions of novel proteins in the GI and faeces of mammals. This is reflected in different and contradicting views on IVD studies in guidance documents and the scientific literature. More complex models have therefore been proposed by some authors. Neither the Simulated Gastric Fluids (SGF) and Simulated Intestinal Fluids (SIF) models nor any of the more complex in-vitro models have, however, been validated so far.

Animal toxicity studies are recommended on a case-by-case basis and might not be required if other evidence suggests that the protein is safe. Toxicity studies might be required and the selection of endpoints might be influenced if

- i. the specificity and biological function/mode of action of the protein is partly known or unknown or of concern (for instance a protease inhibitor);
- ii. the protein is implicated in mammalian toxicity;
- iii. human and animal exposure to the protein is not documented;
- iv. modification of the primary structure of naturally occurring forms;
- v. when estimated levels of intakes of the new protein indicate adverse effects or
- vi. when the protein is not rapidly degradable.

Toxicity endpoints proposed differ across guidance documents, including acute toxicity or 28-day subacute oral toxicity tests with acute toxicity tests being explicitly disregarded by some. In practice, however, with rare exceptions only single-dose acute toxicity tests were conducted.

90-day whole plant/food subchronic studies in rodents can provide additional safety reassurance in the course of protein toxicity assessment depending on the particular case (design of the study, nature of the protein, GM plant species etc.).

Exposure assessment and risk characterisation should – according to recent guidance documents - follow the same rationale as in other regulatory contexts. Estimates of daily intake based on a comprehensive database for food consumption are only included in recent risk assessment dossiers. In case a more formal calculation of the margin of safety (MoS) is included, a NOAEL is sometimes derived from acute toxicity tests. This does not correspond to normal toxicological practice, which foresees a NOAEL to be calculated based on a minimum of 90-day subchronic studies. Alternatively, it is being argued in risk assessment dossiers that either exposure (e.g. no protein detectable in the food) or toxic properties (e.g. no toxic properties detected in maximum-dose acute toxicity tests) are negligible and therefore no risk is anticipated.

In essence, this review of toxicity assessment practice revealed a number of weaknesses. In order to further investigate the relevance of these findings a review was conducted on proteins known to be toxic via the oral route (to further characterise the potential hazards from proteins). Furthermore, the approach to protein toxicity assessment in the context of GM plants and foods is compared to the assessment approaches for food enzymes and biopesticides in order to identify possible differences which could guide a discussion on improvements and a **comparison of protein toxicity assessment approaches**.

In contrast to the GM plant and food context, a toxicity assessment of food enzymes does not include IVDs and homology comparisons to known toxins. It typically includes a 90-day subchronic oral toxicity test on rodents along with mutagenicity tests on bacteria and an in-vitro test for chromosomal aberration.

The main reason for the differences to the GM plant and food context is that proteins are assessed as fermentation isolates rather than pure proteins. Food enzyme isolates contain a large number of by-products and can include contaminants such as mycotoxins and bacterial toxins. These substances are of greater toxicological concern than the pure enzymes. In the context of GM plants and food such contaminants are not an issue. Very similar considerations are also guiding the toxicity risk assessment of biopesticides from microbial origin. In this case the use of cell or spore suspensions also creates the need for pathogenicity assessment. Overall, food enzymes and biopesticides might therefore be less informative for protein toxicity assessment in the context of GM food and feed.

A review on proteins causing toxic effects via the oral route identified a range of plant and bacterial proteins to be of some kind of concern. Some of these toxins, such as ricin from Castor beans or botulinus toxin from *Clostridium botulinum* belong to the most powerful toxins known. The most widespread adverse effects in case of plant proteins result from lectins and proteinase inhibitors (PI). Lectins are considerably resistant to digestion in the human GI. Following receptor binding in the gut lectins frequently induce hyperplastic intestinal growth affecting the overall energy metabolism and function of the entire GI tract, causing a range of secondary effects. With lectins it seems difficult to distinguish between toxic and anti-nutritive effects. PIs are reducing the efficiency of digestion; their long-term consumption can result in continuous overstimulation and growth of the pancreas.

Bacterial toxins generate their adverse effects either by acting on the target cell surface and irreparably damaging the cell membrane, altering normal cellular signal transduction, or by exhibiting enzymatic activity in the cytoplasm. Overall pathogenic symptoms may be: abdominal pain, headache, bloody diarrhoea, nausea, vomiting and fever. Types of toxins involved in food poisoning are emetic toxins, neurotoxins and enterotoxins. Emetic toxins cause vomiting by binding specific receptors in the duodenum. Neurotoxins are proteins which act on the nervous system and enterotoxins are defined as proteins that are active in the intestine and cause diarrhoea. Enterotoxins are either disrupting the cell membrane causing cell death or they are cytotoxic enterotoxins, which enter the epithelial cell and cause diarrhoea without direct membrane disruption. From this review it appears that bacterial toxins tend to cause acute effects whereas lectins and perhaps PIs could also cause long-term adverse health effects including toxic effects.

In the regulatory context the concerns with protein toxicity have so far focused on acute effects only. Short-term and long term toxicity tests conducted in the regulatory procedure have not revealed causes for concern. However, these tests have been conducted on a small number of food enzymes only and might not be considered representative of a broader range of proteins.

The review and analysis allows to draw some overall conclusions:

- The approach in protein toxicity testing in the context of GM plant and food relies strongly on ex-vivo studies as there is either no compulsory (EU) or only acute toxicity testing (USA, Australia). Evidence from this study should alert the risk assessors and trigger further studies.
- Evidence from the scientific literature and regulatory toxicology does not indicate a reason for immediate concern. However, there does not seem to be a substantial database on protein toxicity.
- As described above there are a number of weaknesses associated with the individual methods and types of evidence considered. Furthermore, it remains unknown if this approach would reliably work, as experience is so far limited to a small number of more familiar proteins preselected for the use in food/feed - thus there has been no test case to challenge this approach. The practice of using microbially produced proteins for animal studies and of envisaging acute adverse effects only adds considerable uncertainties to the currently used approach in protein toxicity assessment.

From this analysis, proposals for further research, guidance and need for clarification are derived:

Further **research** would particularly be needed

- To clarify in greater detail any toxic effects of proteins including effects beyond acute effects

- To clarify the relevance of IVD for the in-vivo situation in humans and possible alternatives
- To clarify the fate in and absorption of proteins from the GI tract of humans

More detailed **guidance** and **re-consideration** would be needed on

- Currently applied IVD studies (laboratory protocol, chemicals used, validation)
- Homology searches (databases, parameters, verification, quality assurance, updates)
- The robustness of evidence to which one could legitimately refer if relying on the history of consumption/exposure; and the acceptability of distant relationships between the protein with a history of exposure and the test protein

Clarification would be needed

- To differentiate more clearly between toxic and anti-nutritive properties of proteins
- On the explicit need and methods to assess possible anti-nutritive properties alongside toxic properties of novel proteins

As long as the protein toxicity assessment approach has neither been tested nor improved it might be considered to conduct repeated-dose tests according to OECD protocols (with the duration depending on the evidence available) as a standard requirement for assessing novel proteins.

Part B: Assessment of ecotoxicity

Part B analyses the current approach in ecotoxicity assessment of GMP expressed proteins. The aim of this review is to investigate and discuss the assumptions underlying ecotoxicity assessment and the currently applied approaches in ecotoxicity assessment of newly expressed proteins in genetically modified plants as well as to provide suggestions for improvements. Additionally, general questions on the mode of action, specificity and ecotoxicity of plant, fungal and bacterial proteins and specifically on Cry-proteins as expressed in GMPs are addressed. Notifications of GMPs for cultivation within the EU, relevant regulatory and guidance documents as well as the published literature are the basis of this review.

Proteins introduced into commercially relevant GMPs for combating insect pests are mainly restricted to Cry-proteins derived from the common soil bacteria ***Bacillus thuringiensis***. The current practice of ecotoxicity risk assessment of Cry-proteins expressed in GMPs is based on specific **assumptions** which underlie the assessment approach in GMP notifications. Cry-proteins are generally believed to act as acute toxins only, to be highly specific against a certain target pest, to be rapidly degradable in soil ecosystems and not to interact in case of more than one protein being expressed in the same GMP. Consequently, acute, short-term ecotoxicity tests with few non-target species and short-term laboratory studies to estimate protein degradation in soil are provided by notifiers in GMP notifications. Assessments of exposure and risk characterisations for non-target organisms are only rarely provided in GMP notifications.

Cry-proteins in GMPs differ to a variable extent from the native Cry-proteins with respect to amino acid length, amino acid sequence and protein size. Cry-Proteins are frequently expressed in bacteria other than the native bacteria (e.g. in ***E. coli***) when used for ecotoxicological assessment and may differ from the protein expressed in the GMP. Different fragment sizes of the proteins between plant and microbially expressed proteins are often observed. Consequently, uncertainties remain as to whether these differences also result in differential toxicities towards target and non-target organisms if microbially produced proteins are used as **test substances** in ecotoxicological assessments.

Ecotoxic proteins produced in plants, bacteria or fungi can have either narrow or broad specificities depending on the protein and the insect species tested. There is evidence that the **specificity** of a certain insecticidal protein can vary considerably between species even if they are closely related. Suggestions for classifying the specificity of a certain ecotoxic protein according to the ecology of a species are controversial and it remains unclear whether specialists are better adapted to a specific ecotoxic protein or whether generalists show more tolerance against a wider range of toxic proteins. However, it seems clear that the taxonomic position is largely not responsible for the reaction elicited in a certain insect species. It is also evident that a range of compensation mechanisms has evolved in insects such as additional or changed secretion of enzymes, as for example in the case of proteinase inhibitors. Consequently, the specificity of a certain ecotoxic protein cannot be assumed a priori. This has consequences for tests in GMP notifications generally using a few commercially relevant species.

Additionally, several factors relating to the characteristics of the toxin, conditions in the insect gut as well as environmental conditions such the presence of other chemical compounds or relevant nutrients in the plant or the general health status of an insect can considerably influence the toxicity and therefore specificity of Cry-proteins. There are also indications for a wider specificity of ecotoxic proteins in general and specifically Cry-toxins than previously assumed. Uncertainties remain regarding the exact mode of action and the causes of toxicity of Cry-proteins as well as with respect to degradation and their remaining toxicity when Cry-proteins pass through organisms to higher trophic levels.

Exposure of non-target organisms to Cry-proteins expressed in GMPs is in most cases not considered to be relevant in GMP notifications. Only few GMP notifications contain an assessment of exposure to Cry-proteins for a limited range of organisms. Shortcomings of these assessments are the use of a few functional groups of organisms and expression values of the respective protein in the GMP derived from non-EU field trials as a basis to estimate the predicted environmental concentration. Exposure pathways other than the direct feeding of non-target organisms on the plant during cultivation are generally not included. There is evidence that a range of non-target organisms are actually exposed to the Cry-proteins of GMPs in the field and Cry-proteins have been shown to pass through the food chain being available for higher trophic levels. Furthermore Cry-proteins derived from GMPs have been shown to be present in a range of environmental compartments where they may persist.

The **ecotoxicity assessments** of Cry-proteins provided by the notifiers in GMP notifications have limited significance for the cultivation of GMPs in an agricultural environment. Generally, these toxicological tests are carried out with a limited set of insect taxa. The insect species used in most notifications comprise honey bees, green lacewing larvae, parasitic hymenoptera and ladybird beetles. Infrequently, tests with earthworms or Collembola are included. Tests with higher organisms such as birds or mammals, tests using water organisms (daphnids) or other species such as butterflies or ground beetles were conducted only in a few cases. These studies generally intend to assess acute toxicity with adult organisms and the microbially produced proteins as test substances applied with an artificial laboratory diet for a short testing period.

These studies are often not conducted according to standardized test protocols and consequently their validity is not guaranteed. High mortalities in control groups often limit the testing period, and the concentrations used are not comprehensible or the exposure of the test species to the test substance is not guaranteed. Positive control groups are often not included. Parameters such as concentration or length of the test vary considerably between studies using the same test organism. The validity of results

obtained from such tests is further compromised due to small sample sizes used in these tests, thereby not guaranteeing sufficient statistical power. The toxicological parameters assessed are predominantly acute toxicity signs. In many cases a detailed description of the assessed parameters is lacking.

The use of a limited set of species for the assessment of ecotoxicity of Cry-proteins as currently done in GMP notifications ignores the particular plant-environment interaction when a GMP is released into a specific environment. Although the selection of non-target species for ecotoxicity assessment and the criteria used for their selection are still controversially discussed it is clear that the consideration of a particular environment is required as it corresponds to the case-by-case principle outlined in the risk assessment approach of Directive 2001/18/EC. Additionally, there is evidence that insect species can experience sublethal effects when exposed to insecticidal proteins produced by plants, fungi and bacteria including Cry-proteins, which is not considered in the current approach assessing acute toxicity only. Short-term exposure of non-target organisms gives limited information on the toxicity of a GMP produced protein when organisms can be exposed during their entire life-time or when even multiple generations of a species may be exposed. Life-long or multi-generational testing and the inclusion of different life stages, weakened or diseased organisms as well as the assessment of sublethal parameters would therefore give more realistic indications of the ecotoxicity of these proteins. For a realistic exposure scenario the use of the whole GMP complementing bacterially produced proteins as test substances is crucial in order to avoid differences in protein structure, size and possibly function between GMP and microbially produced proteins, and to consider additional stressors such as secondary plant compounds at the same time.

An assessment of **protein degradation in soil** is frequently contained in GMP notifications for cultivation within the EU. Soil persistence varies considerably with the experimental setup chosen in laboratory experiments and depends on environmental factors such as soil type and temperature. The currently used approach of short-term assessment of microbially expressed Cry-proteins in only one soil type under laboratory conditions has considerable shortcomings with respect to the methodology used, the models underlying protein degradation in soil and the conditions applied. Low-level and prolonged persistence of Cry-proteins in soil under field conditions and the presence of these toxins in water, as indicated by published literature, cannot be assessed appropriately with the currently applied methods. Several soil types, whole GMPs instead of isolated proteins and biochemical assessment parameters need to be taken into consideration when the fate and behaviour of these proteins in different environmental compartments needs to be assessed.

Notifications of GMPs expressing more than one Cry-protein (**stacked event GMPs**) generally lack an exposure assessment and refer to the toxicity and protein degradation assessments of the respective single event GMPs. This approach is considered to be justified by the notifiers as interactions of Cry-proteins against target or non-target organisms are considered to be unlikely. Both synergistic and antagonistic effects of insecticidal proteins including Cry-proteins have been described in the literature. Consequently their assessment is of high relevance for GMPs expressing more than a single insecticidal protein.

The analysis of the practice of ecotoxicity assessment of proteins as expressed in GMPs reveals a range of weaknesses as outlined above. Relevant EU regulations and guidance documents for the placing on the market of a GMP do not give detailed guidance on the ecotoxicity assessment. However, delayed and long-term as well as indirect effects are explicitly addressed in EU environmental risk assessment requirements.

In the US GMPs expressing Cry-proteins are considered biopesticides. The regulatory requirements focus on the assessment of the introduced protein itself and not the whole GMP. Consequently, the microbially produced protein and a standard set of test organisms as well as a standard test for persistence of the protein in the soil are used in ecotoxicity assessments. Consequently, notifiers of GMPs in the EU adapt to US regulatory requirements with respect to tests to be applied, test organisms to be chosen and toxicological endpoints to be tested. In contrast to GMPs the risk assessment approach for microbial plant protection products in the EU is much more detailed and includes the assessment of the fate, behaviour and persistence of these products in different environmental compartments. Another marked difference is that the risk characterisation is based on an exposure and effects assessment for selected test organisms, the specification of trigger values for authorisation and the recommendation of standardized test protocols.

The following limitations of the current assessment approach to determine the ecotoxicity of GMP expressed proteins have been identified and the following suggestions are made:

- The lack of a systematic exposure assessment of non-target organisms in GMP notifications ignores the availability of Cry-proteins to non-target organisms via direct or indirect feeding or through other exposure pathways such as water, sediments or soil. Improvements should be made with respect to exposure assessment and risk characterisation, as is the case in other regulatory contexts such as microbial plant protection products.
- Major limitations are identified in the currently applied tests on ecotoxicity and protein degradation with respect to test design, methodology and validity of the test. The current approach does not consider evidence of protein toxicity other than acute as well as protein persistence other than short-term. At the EU level clear guidance is needed for notifiers with respect to the selection of test species, methodology used and parameters assessed.
- Uncertainties are evident with respect to the specificity and biological activity of the Cry-proteins used as test substances. Thus the selection of test substances should consider field conditions and include whole GMPs in order to account for differences between plant and bacterially produced proteins as well as host plant composition.
- Currently, in GMP notifications standardised tests are rarely used for ecotoxicity assessments. The use of standardised test protocols (e. g. OECD) is, however, crucial in order to guarantee valid test results. Additionally, an adaptation of existing test protocols or the development of new protocols specifically for the use of protein ecotoxicity assessment in GMPs is necessary.
- Possible interactions of ecotoxic proteins expressed in the same GMP should not be dismissed but included in ecotoxicity assessments.

From the above analysis the following research needs can be derived:

- Uncertainties regarding the specificity and mode of action of Cry-proteins against target and non-target species are evident in general and especially with respect to the proteins used in ecotoxicological tests (expressed in GMPs or bacteria). Research is needed to improve the current understanding of Cry-protein ecotoxicity.
- The data basis on the fate and behaviour of Cry-proteins in different environmental compartments or when passing through the food web is still very limited. With the large scale introduction and cultivation of GMPs these proteins will be present in many habitats. Thus a thorough understanding of their behaviour and persistence in these environments is crucial.

Introduction and motivation

The risk assessment of genetically modified plants (GMPs) and foods and its underlying concepts and assumptions have been a matter of controversy in the regulatory and scientific domain since more than two decades. A previously undertaken in-depth analysis of risk assessment dossiers submitted between 1996 to 2001 identified inconsistencies and shortcomings in EU risk assessment practice (Spök et al. 2002, 2003, 2004). This analysis also pointed to particular uncertainties associated with the approach and methods used in toxicity assessment. Possible toxic properties are being assessed in two different ways: (i) assessment of any novel proteins introduced into the GM plant as well as deliberately introduced novel metabolites; and (ii) assessment of the whole plants /whole food. The latter aspect is related to approaches and methods to detect potentially adverse side effects (pleiotropic effects) of the genetic modification. This aspect has been a focal issue in the debate since very long and is being considered by a wealth of scientific literature on the concept of and methods to establish substantial equivalence, on novel profiling methods to detect variations in protein, metabolites and RNA expression patterns and on animal feeding studies using whole plant/whole food extracts (herein referred to as whole plant/food feedings studies). Especially the relevance and feasibility of whole plant/food feedings studies have sparked discussion among toxicologists and has recently become the main focus of public consultations and guidance documents (e.g. ILSI 2003, EFSA 2007).

Overshadowed by these issues protein toxicity assessment so far received less attention. However, there are reasons why toxicity assessment of novel proteins deserve attention.

First, from the preceding study mentioned above it became evident that protein toxicity assessment in the course of GM plant risk assessment is much more relying on indirect evidence. Amino acid sequence comparisons to known toxins, in-vitro digestibility studies, acute toxicity studies in rodents along with evidence of a history of safe use is the evidential basis of protein safety compared to other regulatory contexts such as chemicals, pesticides, and food additives. Moreover, shortcomings in the methodologies used, the way tests are conducted and the soundness of evidence provided became apparent. This has to be considered against the backdrop of a surprisingly small knowledge base on toxic properties of proteins. Given the broad scope of this previous analysis covering toxicity assessment, allergenicity assessment and substantial equivalence it was, however, beyond the scope to work on the very detail of approaches and methods.

Second, despite the focus on whole plant/food feeding studies and profiling methods toxicity assessment will continue to play an important role in GMP toxicity assessment. The novel protein represents in any case a novel substance in the plant/food. As with other single compounds deliberately or accidentally present in food, the whole range of well established animal and in-vitro toxicity tests can in principle be conducted on every introduced novel protein. So far, only a small number of proteins have been introduced into GM plants authorised for commercial purposes, with the majority of plants containing modified or mutated **cry**, **epsps**, and **pat** genes conferring insect resistant or herbicide tolerance. Given the targeting of staple crops and the increasing adoption of this first generation GM plants in agriculture consumer intake of some of these novel proteins is likely to increase and might simultaneously occur from several plant sources. The variety of introduced proteins along with the degree of their novelty to the food chain is also likely to increase with subsequent generations of GMPs.

GM crops for industrial use might have much higher expression rates (e.g. in case of maize 3272 expressing a thermostable amylase from **Archaea**). This kind of GM plants

are not intended for food use but given the possibility of accidental admixture food safety assessments are likely to be required. Even more emphasis will be on protein toxicity assessment if the use of food/feed crops for production of biopharmaceuticals and other industrial substances on open fields will increase and enter the commercial stage.

Protein toxicity assessment in the context of GMOs is normally understood as human (mammalian) toxicity only. Assessment of possible adverse effects of novel proteins on soil organisms or other non-target species are usually included in the context of environmental risk assessment. A review of risk assessment dossiers from 1995 to 1998 revealed in particular the absence of animal testing beyond acute toxicity tests and feed conversion studies (Spök et al. 2002). Scientific reports on the persistence of novel proteins in the ecosystem and on potential adverse effects on soil and other non-target organisms have been acknowledged as relevant by risk assessors. Although risk assessment practice has been changing since the end of the 1990ies, it has not been systematically investigated so far.

Methodology

Against this backdrop the overall goal of this study is to critically evaluate protein toxicity assessment including ecotoxicity in the context of GM plants and food, to identify shortcomings and weaknesses and to develop proposals for further improvement.

The study investigates the knowledge database on protein toxicity and assessments approaches and methods applied for proteins. Protein toxicity is not a well defined area of scientific research, toxic proteins are dealt with in several contexts such as human and veterinary medicine, toxicology, pharmacology, food and feed safety, nutrition sciences, biopesticides etc. Knowledge of and experience in protein toxicity assessment is dispersed across several regulatory contexts, in particular food enzymes, feed enzymes, biopesticides, chemicals and biopharmaceuticals. A major task in the course of this study was to compile evidence from these various fields and contexts. For reasons of feasibility, relevance and focus the scope was eventually limited to include food enzymes and biopesticides only, as the characteristics and exposure is similar compared to GMOs and will therefore be more relevant compared to other regulatory fields. The study thereby builds on the inventory of protein toxicity assessment practice in risk assessment of GM plants and food resulting from earlier work (SPÖK et al. 2002, 2003, summarised in SPÖK et al. 2004).

Evidence and regulatory practice from these fields provided reference points for a critical discussion of protein toxicity assessment in the context of GM plants and food. The analysis and recommendations is based on a thorough review of the scientific literature, regulatory documents, and expert interviews. A draft version of this report was critically discussed in the course of an expert workshop. The following experts attended the workshop and contributed to the discussion: Susan Bardocz, Mandy Bruch, Michael Freissmuth, Corinne Herouet-Guicheney, Angelika Hilbeck, Daniela Jölli, Theresa Hall-Manning and Arpad Pusztai. The comments obtained from this workshop along with extra comments received from non-participating experts were then used to revise the report. Extra comments were obtained by: Morten Poulsen and Deepak Saxena.

The report has two main sections, Part A ***Assessment of toxic and anti-nutritive properties for humans*** and Part B ***Ecotoxicity Assessment***. Part A is structured as follows. Chapter 1 provides an introduction into toxic proteins and protein toxicity. Chapter 2 describes and critically discusses protein toxicity assessment in the context of GM plants and food. Chapter 3 describes protein toxicity assessment in the context of food enzymes and biopesticides. Both Chapters are based on regulatory guidance and practice as well as on relevant scientific literature. Chapter 4 provides a discussion of focal issues identified in the preceding section. Chapter 5 draws overarching conclusions and provides recommendations for further improvement of protein toxicity assessment. In the course of the project it became evident that it can be difficult to draw a clear boundary between toxic and anti-nutritive effects in animal studies. Therefore, anti-nutritive properties were included in the scope of Chapter 1 and 4.

In Part B Chapter 7 provides an outline of ecotoxic proteins from several sources such as plant, fungal and bacterial proteins. Chapter 8 gives an overview of known mechanisms of actions of ecotoxic proteins from these sources. In Chapter 9 ecotoxic effects of proteins as described in the literature are reviewed. Chapters 10 and 11 give an overview of the ecotoxicity assessment in the context of microbial plant protection products and GM plants. The practice of ecotoxicity assessment of the proteins as expressed in GM plants intended for cultivation is described in Chapter 12 and assumptions and methods in ecotoxicity assessment of GM plants are critically

discussed in Chapters 13 and 14. Chapter 15 summarizes conclusions drawn from the preceding chapters and suggests possible improvements.

In order to improve both readability and handling of this report it was decided to include the more comprehensive tables which were compiled in the course of the analysis in two separate documents (Supplement Volume and Confidential Supplement Volume). The Supplement Volume can be obtained through the editor (www.gentechnik.gv.at).

Part A: Assessment of toxic and anti-nutritive properties for humans

1 Toxic Proteins – Protein Toxicity

Compared to the 2.3 million proteins that have so far been registered in protein databases (SONNHAMMER et al. 1998, FINN et al. 2006) the number of proteins known to have toxic properties is very small. Nevertheless, some of the most potent toxins known are proteins.

This Chapter provides background information on proteins known to be toxic to humans, their sources and mode of action. The focus lies on proteins which are toxic via the oral route. Basically these are plant or bacterial toxins which can be consumed incidentally or with food. Some plant toxins are present in food crops or fruits which have to be processed to inactivate the toxin. Some of the proteins known to have adverse effects on human health would be considered anti-nutrients rather than toxins, with others the type of adverse effects which can be observed in animal studies might depend on the dose. This is especially true for lectins. As it could be difficult to draw clear-cut boundaries between toxic and anti-nutrient effects this Chapter occasionally refers to proteins with anti-nutritive properties.

Section 1.1 focuses on plant toxins, section 1.2 on bacterial toxins. Some of the most potent toxins are of animal origin, e.g. from snakes, spiders and sea organisms. These toxins are frequently acting at the cell surface by interfering with the most important ion channels and are usually toxic if applied parenterally. They are, however, of little concern if consumed as cooked food. Furthermore, most of the animals producing toxins do not represent a major part of the human diet. Therefore animal toxins are not considered in this Chapter. Section 1.3 provides a summary and a brief discussion of the relevance to the topic of the report.

1.1 Toxic proteins from plants

Major classes of protein based plant toxin include proteinase inhibitors (PI), antimicrobial peptides, ribosome inactivating proteins and lectins (CRAIK et al. 2002). Their toxicity against mammals (humans) can be understood as a consequence of forming part of a self defence mechanism of the plant. The following table gives selected examples of protein based plant toxins.

Lectins: Lectins are a heterogeneous group of proteins able to bind to carbohydrates (sugars). They either act as single proteins (monovalent lectins) or in combination with a ribosome inactivating component (RIPs). Monovalent lectins are only “mildly” toxic (anti-nutritive) compared to RIPs, but their effects are generally of great importance for nutrition, since they form part of important human alimentation plants. Mode of action and effects are therefore extensively described in the following chapter.

Ribosome Inactivating Proteins (RIP): These are plant defence enzymes that specially and irreversibly inactivate eukaryotic and prokaryotic ribosomes and inhibit viral replication. There are two types of RIPs: Type 1 RIP is not cytotoxic in-vivo but highly potent in-vitro. Reason for its in-vivo inactivity is because type 1 RIP lacks a cell binding component (i.e. a lectin). Though such a lectin forms part of a type 2 RIP facilitating the entrance into host cells and raising high in-vivo toxicity.

Protease Inhibitors (PI): Proteinase (trypsin, chymotrypsin) inhibitors have been isolated from a variety of plants (see Table 1). They have various functions within the plant, examples are regulation of metabolism, storage, defence against micro-organism and insects. The latter property makes them interesting for pest applications.

Antimicrobial Peptides (AMP): Antimicrobial peptides represent a large class of protein based plant toxins with low molecular weight. They form part of defense mechanism for instance and not only occur in plants, but also in mammals. In plants they comprise thionins, plant defensins, hevein-type and knottin-type antimicrobial proteins, lipid transfer proteins, permatins and chitinases. They are active against a large spectrum of microorganism, including bacteria and filamentous fungi and considered to be “natural antibiotics”.

Antimicrobial peptides are not further considered since they act against bacteria and fungi.

Table 1: Selected examples of protein-based plant toxins.

Class	Examples	Plant Origin	Toxicity / mode of action
Protease Inhibitor	BBI (Bowman-Birk soybean proteinase inhibitor)	Soybean	Trypsin and chymotrypsin inhibitor
	SFTI (sunflower trypsin inhibitor)	Sunflower	Trypsin inhibitor
	PIN-II (potato proteinase inhibitor II)	Potato	Trypsin and chymotrypsin inhibitor
	NaPI (<i>Nicotiana glauca</i> proteinase inhibitor)	Tobacco	Trypsin and chymotrypsin inhibitor
Antimicrobial Peptides	Rs-AFP (Raphanus sativus antifungal protein)	Radish	Antifungal
	MIAMP1 (<i>Macadamia integrifolia</i> antimicrobial protein)	Macadamia nuts	Antifungal
	Pth-St1 (Pseudothionin <i>Solanum tuberosum</i> 1)	Potato	Antifungal and antibacterial
	Ct-AMP1 (<i>Clitoria ternatea</i> antimicrobial protein)	Butterfly pea	Antifungal and antibacterial
Ribosomal Inactivating proteins (RIP)	Ricin	Castor oil plant	Inhibits protein synthesis of eukaryotic 60S rRNA
	Trichosanthin	Serpent cucumber	Inhibits eukaryotic ribosomes, used as an abortifacient in Chinese folk medicine
Lectins	Concanavalin A	Jack bean	Agglutinate red blood cells, insecticidal and larvicidal, induce production of phytoalexins
	PHA (<i>Phaseolus vulgaris</i> phytohemagglutinin)	Kidney bean, French bean	Agglutinate red blood cells, mitogenic activity, antinutrient effects (cause weight loss in animals)
	Soybean lectin	Soybean	Induces gut and pancreas growth

Source: CRAIK et al. (2003), extended

Table 2: Plant lectins.

Example	Acronym	Origin	Specific to
Peanut agglutinin	PNA	<i>Arachis hypogaea</i> (peanuts)	β -D-galactose
Convanavalin	Con A	<i>Canavalia ensiformis</i> (jackbeans)	α -D-Glucose α -D-Mannose
<i>Dolichus biflorus</i> Agglutinin	DBA	<i>Dolichus biflorus</i> (horsegum type)	N-Acetyl- α -D-Galactosamin
Soybean agglutinin	SBA	<i>Glycine max</i> (soybeans)	N-Acetyl- α -D-Galactosamin- β -D-Galactose
Lens culinaris agglutinin		<i>Lens culinaris</i> (lentils)	α -D-Glucose
<i>Phaseolus</i> agglutinin I	PHA - i	<i>Phaseolus vulgaris</i> (common beans)	N-Acetyl- α -D-Galactosamin
Pealectin-I	PSA	<i>Pisum sativum</i> (peas)	α -D-Glucose
<i>Ricinus communis</i> agglutinin	RCA 120, RCA 60	<i>Ricinus communis</i>	β -D-galactose, N-acetyl-alpha-D-galactosamin
Wheat germ agglutinin	WGA	<i>Triticum vulgare</i> (wheat germ)	N-Acetyl- β -(1,4)-D-glucosamin; Chitin, Chitotriose

Source: BROWN & HUNT (1978)

1.1.1 Mode of action and adverse effects arising after oral uptake of plant proteins

1.1.1.1 Trypsin Inhibitors

Proteinase inhibitors inhibit the action of digestive proteinases such as trypsin/chymotrypsin. Ingestion of raw soybean for example reduces proteolysis of dietary protein, causes increased secretion of pancreatic enzymes and impairs body growth of laboratory species. Feeding experimental animals on diet containing isolated soybean trypsin inhibitors caused insignificant growth depression in rats and chicks, but induced enlargement of the pancreas in rats, chicks and mice but not in pigs, dogs, calves, monkeys and presumably humans (BIRK 1996). Prolonged feeding of rats and mice with a diet rich in potato and soybean trypsin inhibitor produced short term pancreatic hyperplasia in both species and long-term nodular hyperplasia (enlargement) and acinar adenoma in rats (D'MELLO 2003). Most information of the nutritional effects of trypsin inhibitors come from experiments with animals (pancreas enlargement). While some species are sensitive to that noxa (rats, chicks), others are not (pigs, dogs, calves).

Because of this difference in species response, any prediction in regarding the effect of trypsin inhibitors in humans is uncertain. (D'MELLO 2003). Short term experiments with humans indicate that the human pancreas is responsive to trypsin inhibitor (LIENER 1986).

Trypsin inhibitor of soybeans have received the most study, but protease inhibitors are widely distributed in nature and are present in many common food components of human diet, including not only most legumes but also most of the cereal grains, potatoes and eggs. Trypsin inhibitors are inactivated by heat treatment, however commercially available edible soybean products retain 5–20 % of the trypsin inhibitory activity present in the original soybeans (LIENER 1986).

1.1.1.2 Plant lectins¹

High levels of lectins are found in various plant tissues of legumes (beans including soybeans and peanuts), grains (cereals) and plants from the nightshade family. Lectins very specifically interact with certain carbohydrates, as they bind to polysaccharides, glycoproteins or glycolipids (free or bound to cell membranes) in a kind of antigen – antibody reaction.

One of the nutritionally most important features of plant lectins is their ability to survive digestion by the human gastrointestinal tract thus allowing the lectins to bind to membrane glycosyl groups of the cells of the digestive tract.

When orally administered to test animals they bind to, perturb and damage intestinal enterocytes, and reduce the absorptive surface of the small intestine, which undergoes hypertrophy (= increase of the size of an organ) and hyperplasia². One of the possible explanations of the toxicity of the lectins are changes in membrane properties induced by binding of lectins.

As a result of this interaction a series of harmful local and systemic reactions are triggered placing this class of molecules as antinutritive and/or toxic substances. At high intakes that has effect on growth, body weight and health of consuming animals.

Systemically they can disrupt lipid, carbohydrate and protein metabolism, promote enlargement of key internal organs and tissues and alter the hormonal and immunological status (VASCONCELOS & OLIVEIRA 2004).

Lectins are also endocytosed by intestinal cells and enter the circulation bound to unidentified blood cells.

The pathological changes (i.e. multiple histological lesions) in animals injected with kidney bean extracts may be attributed to a reduced uptake of essential amino acids or a reduced intestinal absorption of glucose. Diet rich in raw soybean has a goitrogenic effect (resulting in difficulties for the thyroid gland producing hormones). When raw ground garden beans are supplemented with essential nutrients diet, weight loss and death of rats occur within 1–2 weeks due to lectin toxicity. Raw soybean meals reduces fat and fatty acids absorption in young chicks or the utilization of vitamin D in turkey. These effects are not found if the soybeans are heated before. Raw navy beans have been found toxic for Japanese quail but not for germ-fed birds. Several cases of human intoxication were reported due to ingestion of raw or partially cooked beans.

Many common food plants contain lectins that compromise intestinal integrity and interfere with intestinal absorption. These include lectins from soybeans, jackbeans, pea, and red lentils.

So far, little is known about the function of lectin in plants, yet their wide distribution in all tissues of plants and their ubiquitous presence in the plant kingdom suggest important roles for these proteins. It is suspected that they play a role in binding nitrogen-fixing bacteria to legume roots and they were shown to have fungitoxic, anti-insect and anti nematode properties.

¹ The terms phytohemagglutinins, phytagglutinins are used synonymously.

² Hyperplasia (or "hypergenesis") is a general term referring to the proliferation of cells within an organ or tissue beyond that which is ordinarily seen. Hyperplasia may result in the gross enlargement of an organ, the formation of a benign tumor, or may be visible only under a microscope. Hyperplasia is considered to be a physiological response to a specific stimulus, and the cells of a hyperplastic growth remain subject to normal regulatory control mechanisms.

Furthermore, there is a remarkably high resistance to proteolysis and stability over a wide pH range, even when they are out of their natural environment. It has also been observed that far lectins are even more resistant to proteolytic digestion in-vivo. This may be a result of binding to epithelial cells in the gut thus protecting against enzymatic degradation. Lectins which are not bound to the mucosa usually induce little or no harmful antinutritive effect for consumers (PUSZTAI 1996). The physiological mechanisms as well as consequences of undigested lectins on biological activities in higher animals are shown in Table 3 (VASCONCELOS & OLIVEIRA 2004).

Table 3: Mode of action and effects of lectins.

Mechanism	Effect	
Binding to Gut cells	Increase cell turnover	Nutrient malabsorption Impaired immunological effects Poor growth Death
Interaction with enzymes	Poor protein digestibility	
Non-available amino acids	Negative N balance	
Changes in organs (i.e. small intestine)	Alteration in metabolism	
Mucottractive effects	Draining of energy	

Systemic effects: An anatomical change evident in rats fed on lectins is the severe atrophy of the thymus, further pancreas hypertrophy and liver enlargement after feeding animals with lectin is observed (VASCONCELOS & OLIVEIRA 2004). Disturbances of hormonal homeostatis of animals were observed upon feeding lectins, i.e. reduced circulating levels of insulin, while the exact mechanism of the reduction remains unclear. Generally lectins have the potential to interfere with metabolism and cause morphological changes in the gut.

But there are also secondary effects which are not well understood until now: Lectins promote proliferations of coliforms, mainly *E. coli* in the small intestine (PUSZTAI et al. 1995). As a consequence bacterial toxins may become a secondary problem for animal health.

Patients suffer from profuse vomiting and diarrhoea. Yet lectins differ in their toxic potential, not all of them are orally toxic and only few can be fatal if ingested in high amounts. The mechanisms by which lectins mediate toxicity and the characteristics which dictate whether a lectin will be deleterious or not are not completely understood. In animal feeding studies with diets containing plant lectins (e.g. uncooked soy beans) the symptoms are loss of appetite, decreased body weight and eventually death. It has been proven, that from intradermal or intraperitoneal administration the orally toxic potential cannot be concluded. Thus, the only reliable way of predicting oral toxicity of lectins is by feeding different types of target animals on diets containing the purified lectin. Data indicate, that multiple exposure as well as long term toxicity studies are necessary to address lectin toxicity.

Several lectins are resistant to proteolytic digestion, e.g. PHA, wheat-germ agglutinin, tomato and navy bean lectin. Dry heat may also not completely destroy lectins, since hemagglutinating activity is found in processed wheat-germ, peanuts and dry cereals.

1.1.1.3 Ribosome inactivating proteins (RIP)

There is a variety of cytotoxic plant derived proteins which are related to each other in function and structure. They inhibit protein synthesis by specifically and irreversibly inactivating eukaryotic ribosomes, therefore they are called "ribosome-inactivating proteins" (RIPs).

Examples for one type of RIPs are the lectins ricin and abrin. The glycosylated proteins consists of domains A and B connected by disulfide. The A chain is a glycosidase, which removes an adenine residue from ribosomal RNA and the modified ribosomes are unable to support protein biosynthesis. While the B chain – a lectin - binds to the cell surface, chain A enters the cytosol and blocks protein synthesis by removal of one adenine from a ribosomal subunit (OLSNES 2004).

Ricin found in the seeds of the castor bean plant (***Ricinus communis***)³. Ricin has no effect on prokaryotic (bacterial) ribosomes. Poisoning takes place by ingestion of the castor bean, the symptoms start within a few hours following ingestion: abdominal pain, vomiting and diarrhoea. Within several days severe dehydration, decrease in urine and in blood pressure takes place. If the seed is swallowed without chewing and there is no damage to the seed coat, it will likely pass harmlessly through the digestive tract. Only if chewed or broken, the ricin will be absorbed by the intestine.

Ricin, abrin, crotin and related toxins produce similar macroscopic and pathological lesions which are intensive inflammation with destruction of epithelial cells, oedema, hyperemia and hemorrhages in the lymphatic tissues. Several signs of toxicity may include: fatty degeneration and necrosis in the liver, degenerative lesions of the myocard.

For humans the maximum tolerated dose seems to be 40 µg for a person of 60 kg, LD₅₀ for mice (parenteral injection) is 2.6 µg/kg and just one milligram can kill an adult. Ricin is 100 times more toxic when administered parenterally but intoxication is not rare in countries where the plant grows in abundance (OLSNES 2004). The destruction of ricin toxicity by heat is well known. This is done nearly completely by steam heating with preliminary soaking of the castor beans, whereas dry heating has little effect.

The highly toxic protein abrin can be found in the scarlet seeds of ***Abrus precatorius***, which are often used in cheap jewellery. Intoxication of human seems to be rare, since the seeds have a hard shell and may pass the intestinal tract without digestion. Nevertheless it was used for empoisoning humans by applying dried paste with needles under the skin.

Ricin and abrin show extensive similarities both in the A- and the B-chains and again is very similar to A-chain Type 1 RIP. Within the amino acid sequence, Glu 177 and Arg180 are particularly important for the toxic effect and mutation of one of these residues strongly reduce the enzymatic activity. Furthermore, these amino acids are conserved throughout the family of RIP proteins (OLSNES 2004).

Besides, ***Ricinus communis*** and ***Abrus precatorius*** there are a few plants containing type 2 RIP toxins. These are the toxins modeccin from ***Modecca*** or ***Adenia digitata*** and viscumin lectins from mistletoe (***Viscum album L.***).

The function of the toxin in the plants has not yet been fully clarified, since there is not much evidence that they are deterrents against animals or parasites. Another unexpected finding is that toxins from the bacterium ***Shigella dysenteriae*** and certain ***E. coli*** strains have A-chains that are structurally closely related to the ricin A-chain. This raises the question if there was a transfer from or to bacteria. In the same way surprising is the interaction of the toxins with the animal cells (OLSNES 2004).

³ The plant originates from Asia and Africa, in colder climates it grows like bushes. The main product - castor oil - is produced by hot extraction of the beans, a procedure which destroys the toxin. The major part is used as a lubricant.

1.2 Toxic proteins from bacteria

Since diphtheria toxin was isolated in 1888, microbial toxins have been recognized as primary virulence factor(s) for a variety of pathogenic bacteria. Many microbial toxins developed a variety of action paths towards host cells, but nearly all bacterial toxins act inside the cell: Bacterial toxins may be ingested as food contaminants or released in the intestine from microbes contaminating food. Typically bacterial toxins exhibit the following mode of action: Damaging cell membranes and insertion into the target cell after binding and pore forming, inhibiting protein synthesis, activating second messenger pathways, inhibiting the release of neurotransmitters or activating the hosts immune response.

Table 4: Examples for bacterial toxins, their mode of action and related diseases.^a

Organism / toxin	Mode of action	Target	Disease	Toxin implicated in disease
Damage membranes				
<i>Aeromonas hydrophila</i> /aerolysin	Pore-former	Glycophorin	Diarrhoea	(yes)
<i>Clostridium perfringens</i> /	Pore-former	Cholesterol	Gas gangrene	?
<i>Escherichia coli</i> / hemolysin ^d	Pore-former	Plasma membrane	UTIs*	(yes)
Inhibit protein synthesis				
<i>E. coli</i> / <i>Shigella dysenteriae</i> / (Shiga toxins)	N-glycosidase	28S rRNA	HC and HUS	yes
<i>Pseudomonas aeruginosa</i> / (exotoxin a)	ADP-ribosyltransferase	Elongation factor 2	Pneumonia	(yes)
Activate second messenger pathways				
CNF (<i>E.coli</i>)	Deamidase	Rho G-proteins	UTIs*	?
LT (<i>E.coli</i>)	ADP-ribosyltransferase	G-proteins	Diarrhea	yes
<i>Clostridium botulinum</i> / C2 toxin	ADP-ribosyltransferase	Monomeric G-actin	Botulism	?
Activate immune response				
Enterotoxins (<i>S. aureus</i>)	Superantigen	TCR and MHC II	Food poisoning	yes
Protease				
<i>B. anthracis</i> /lethal factor	Metalloprotease	MAPKK1/MAPKK2	Anthrax	yes
<i>Clostridium botulinum</i> / neurotoxins A-G	Zinc-metalloprotease	VAMP/synaptobrevin SNAP-25 syntaxin	Botulism	yes
<i>Clostridium tetani</i> / tetanus toxin	Zinc-metalloprotease	VAMP/synaptobrevin	Tetanus	yes

a) This table includes examples for bacterial proteins known to be toxic to humans. UTI: Urinary tract infection

Source: SCHMITT et al. (1999)

1.2.1 Structure constituting toxin activity

1.2.1.1 A-B toxins

The first essential step in the intoxication by all bacterial toxins is receptor binding at the cell surface. Bacteria toxins usually consist of two domains, commonly designated as A and B (A-B toxins). The B domain is involved in cell binding and penetration, while

the A domain displays intracellular enzymatic activity. They make use of the pathways used by cells for the intake of ligands and nutrients and to remove un-needed ligands and/or receptors from the cell surface. Cells lacking the relevant receptor are resistant to the toxin. The cell binding and uptake takes place via the B part. They turn into toxic only when they become capable of translocating domain A across the membrane into the cytosol. According to their B part, two groups of bacterial toxins can be distinguished: One having a B part composed of two domains (diphtheria toxin, clostridial neurotoxins, etc.) and another having an oligomeric B domain (cholera toxin, shiga toxin etc.). The first group enters the cell via an acidic intracellular compartment. There is evidence that low pH triggers a conformational change that makes these proteins able to insert in the lipid bilayer and to mediate the transfer of the catalytic A domain to the cytosol. The second group of toxins does not need a low pH environment for their entry into the cytosol, and there is evidence that the reduction of the single disulfide bond that links A to B is sufficient to cause a change in the solubility properties of the A chain, so that it becomes able to penetrate the lipid bilayer.

1.2.1.2 Channel forming toxins (CFT)

The channel forming toxins (CFT) are either α -CFTs, if they are predicted to form pores with a helical hairpin, or β -CFTs, if they insert, or are predicted to insert, to form pores comprising a β -barrel (LACY & STEVENS 1998). An important type of channel forming proteins are the Cry proteins from *Bacillus thuringiensis* used as microbial pesticides as well as forming the functional bases of a series of genetically modified plants. The mechanism of action of such Cry proteins involves solubilisation of the crystal in the insect mid-gut, proteolytic processing of the protoxin by mid-gut proteases, binding of the Cry toxin to mid-gut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores. They are discussed in detail in the chapter "Biopesticides".

1.2.2 Modes of action

1.2.2.1 Damaging cell membranes

Many bacteria exotoxins have the capacity to damage the extracellular matrix or the plasma membrane of cells thereby causing defects – pores or holes – within the membrane. They are defined as direct lytic factors as opposed to other toxins having enzymatic properties and able to chemically degrade the membrane. Therefore the non-selective exchange through the plasma membrane, normally controlled and directed by selective pumps, endangers the equilibrium and a permanent leakage can be lethal to the dead of the cell. This may be a consequence of the osmotic effect which leads to a water uptake and swelling as well as due to the loss of vital components.

Examples for pore forming proteins are i) Cholesterol binding toxins from ***Streptococcus***, ***Bacillus***, ***Clostridium*** and ***Listeria*** with a LD₅₀ range from 0.2 to 0.8 μ g (i.v.), ii) α – Toxin from pathogenic strains of ***Staphylococcus aureus***. This toxin oligomerizes on the surface of mammalian cells to form hollow cylinders embedded in the membrane. It causes membrane damage to a variety of cells, i.e. red blood cells and white cells. iii) Melitin produced from honeybees (***Apis mellifera***) with a LD₅₀ dose for mice of 4 mg /kg. iii) Aerolysin from ***Aeromonas hydrophila*** is an example where the toxin is activated from a pre-form by removing 43 amino acids. The removal is accomplished by proteases either from the bacteria or by mammalian proteases such as trypsin, chymotrypsin and furin.

Further steps are binding to cell surface, oligomerization and insertion into the plasma membrane. iv) Hemolysin from ***Escherichia coli*** represents an important but not well defined virulence factor in the pathogenesis of ***E. coli*** infections in humans. It is

assumed that – by causing tissue damage – hemolysin promotes the penetration of bacteria into deeper tissue layers.

1.2.2.2 Inhibiting protein synthesis

The toxins considered are generally inhibiting eukaryotic cellular protein synthesis. For instance, the Diphtheria toxin produced by toxigenic strains of *Corynebacterium diphtheriae* is effective via this mechanism. The toxin – a protein with three domains – binds to the cell surface and is transferred into the cytosol. The domains are responsible for cell surface binding, transmembrane transport and catalytic activity. Once delivered the catalytic domain specifically ADP-ribosylates cellular elongation factor 2 and cell death is caused by the protein production inhibition. Diphtheria toxin is extremely potent in sensitive species (human, monkeys, rabbits or guinea pigs) causing death in doses of 100 to 150 ng/kg. The cytotoxicity is correlated with the number of cell surface receptors, therefore no effects are observed with rat and mice cells, since they don't express the toxin receptor. Other potent bacterial toxins are Shiga toxins from *Shigella* and *E. coli*. In these cases the toxin (A subunit) acts as a glycosidase cleaving an adenine residue from the ribosome. It is assumed that they bind to epithelial cells in the intestine of the infected human or animal, entering them and inhibiting protein synthesis. This leads to cell death and a subsequent loss of intestinal absorptive capacity.

1.2.2.3 Affecting signal transduction

Several toxins are able to interfere with a mode of signal transduction, in which membrane proteins (G proteins) are involved. Interaction of toxins with G proteins for instance may cause a cascade-type effect leading to an intracellular accumulation of the second messenger cAMP. Another mechanism of toxins is to bypass membrane signal transduction and directly generating cAMP.

An example for proteins affecting signal transduction is Cholera toxin from *Vibrio cholerae*, whose toxicity is due to modifying cAMP production in eukaryotic cells. This alters ion transport (increase in chloride secretion and inhibition of sodium absorption) which causes the diarrhoea characteristics of the disease. The cholera toxin consists of A and B subunits held together by non-covalent interaction. The activation takes place by cleaving the A subunit into A1 and A2, which are linked by a disulphide bridge. This is done by a protease specific to *V. cholerae*. Toxins with a similar A-B structure and same mechanism are heat labile toxins from *E. coli*. Again, the mechanism is bonding of unit B to cell surface, proteolytic cleavage and reduction of A to A1 and A2, the insertion of A1 into cytoplasm and interference with signal transduction.

1.2.2.4 Affecting cytoskeleton

The cytoskeleton consists of a fibre network formed of filament systems which are highly dynamic and consist of actin. Rapid structural changes of the cytoskeleton depend on their ability to polymerize and depolymerise. Toxins affecting these systems can be divided into such with and without enzymatic activity and small proteinaceous compounds which bind to the cytoskeleton proteins.

Actin is directly modified by toxins, which ADP-ribosylate the protein thereby inhibiting actin polymerisation and causing depolymerisation of the microfilaments. These toxins are constructed with independent A and B unit, where B is responsible for binding to and translocation into the cell. Beside that, there are also non-proteinaceous toxins interfering with actin. The LD₅₀ for activated C2 toxin (C2I + C2II) is about 5 to 50 ng per mouse (i.v. and i.p.). C2 toxin yet is no clostridial neurotoxin and plays no role in botulism. *Shigella flexneri* is causing bacillary dysentery by entering and spreading

inside host epithelial cells. This involves molecular interactions between bacterial virulence factors and the host cell cytoskeleton.

1.2.2.5 Affecting immune and inflammatory response

Toxins can also target the immune system (IS). For instance bacterial toxins can directly act on various IS cells (T lymphocytes) disturbing cellular functions. There is great variety of modifications, activations or dysregulation of the functions of target immunocytes. This can also include the release of inflammatory mediators.

Cholera toxin is a highly active toxin on the IS network by affecting IgG and IgA response. Enterotoxins are produced from ***Staphylococcus aureus*** and ***Streptococcus pyogenes***. Enterotoxicity (vomiting and diarrhoea) is induced in man and primates by oral ingestion of a few micrograms. Systemic introduction by infection with toxicogenic bacteria leads to a toxic-shock syndrome. Anthrax toxin consists of an antigen, which binds to cellular receptors and which induces translocation the second toxin component – the lethal factor – into the cytosol. Anthrax lethal toxin causes shock-like symptoms observed in systemic anthrax infections by inducing macrophages to overexpress proinflammatory cytokines.

1.2.2.6 Affecting membrane traffic

An effective and intense membrane traffic is essential for life of eukaryotic cells. The most finely tuned exocytic process is the release of neurotransmitter at the presynaptic membrane. Neurotoxins are able to block this traffic.

Tetanus neurotoxin by ***Clostridium tetani*** is the cause of spastic paralysis of tetanus. Activation is done by proteolytic cleavage. Botulinum neurotoxins from ***Clostridium botulinum*** are causing paralysis and are able to enter the cytoplasm of the motorneurons and specifically cleave intracellular targets by a proteolytic mechanism.

This results in a loss of function of the neuroexocytosis machinery and a blockade of neurotransmitter (i.e. acetylcholine) release. LD₅₀ (i.v. and i.p.) are in the range of 0.1–0.5 ng/kg (mouse). Vacuolating cytotoxin by ***Helicobacter pylori***, plays a role in the disease of gastric tumors. Incubated with epithelial cells, the cytotoxin causes cell vacuolization. In-vivo it leads to tissue damage and ulcer formation, exposure to low pH (from 5.5 to 1.5) is sufficient to activate the toxin.

1.3 Summary and discussion

Considering mammalian and human exposure to protein components forming part of their diet the most widespread adverse (i.e. anti-nutritional) effects arise from oral uptake of lectins and proteinase inhibitors (PI) as ingredients of vegetative parts of plants (e.g. beans). This correlates with a relative quantity of scientific research and evidence, but does not necessarily mean that there is an exhaustive knowledge about the overall mode of actions of these components. In contrast, uptake of highly toxic protein agents such as type 2 RIPs (e.g. ricin in Castor beans) happens by accidental intake. Antimicrobial peptides are not further considered here since they act against bacteria and fungi.

Lectins – a group proteins which specifically bind or crosslink sugars (carbohydrates) - are considerably resistant to stomach acid and digestive enzymes and also resist degradation in the small intestine for two reasons⁴: First, lectins have a very compact

⁴ Expert interview; 7th of February 2006

globular structure which provides only few sites for proteolytic attack. Receptor binding to cell membrane glycoproteins also stabilises this globular structure. This is suggested since it can be shown that certain types of lectins can be degraded in-vitro (test tube) with pepsin and pancreatin but are generally resistant to degradation in-vivo (i.e. rat gastrointestinal tract). Yet receptor binding does not seem to be a prerequisite for stability in the gut since it has been shown, that agglutinin from snowdrop bulbs in the absence of receptor binding is not degraded during gut passage (PUSZTAI & BARDOCZ 1996).

The reviewed literature suggests that the mode of action of lectins via oral uptake requires a certain stability of the protein against digestion allowing residence in the gastrointestinal tract. Proteins with specific receptors in the gastrointestinal tract seem to have an enhanced protection against digestion. While receptor binding to specific sugars in the gastrointestinal tract seems to improve stability it is also a prerequisite for causing adverse effects. A fundamental physiological effect of receptor binding of monovalent lectins is a reversible hyperplastic intestinal growth which is induced by binding to the brush border membranes of the small intestine (PUSZTAI & BARDOCZ 1996) affecting the overall energy metabolism and function of the entire digestive tract. This interference with the gastrointestinal tract subsequently causes a broad spectrum of metabolic, systemic or immunological reactions and may also cause a cascade of secondary effects: in case of celiac disease (gluten allergy) the immune system attacks and destroys the villi in the small intestine. Here, the mechanisms are not completely understood. On the other hand a resistance to digestion combined with a non binding to the mammalian gut may only result in an overall excretion without a notable effect (BARDOCZ et al. 1995).

Ribosome inactivating proteins like ricin belongs to the so called type 2 RIPs, where the lectin forms a subunit (B chain) and is attached to a toxic subunit and has to be distinguished from type 1 RIP. The lectin subunit binds to the target cell surface and thus provides a “drug carrier” for the attached toxin. Type 1 RIPs are much less cytotoxic due to lack of such a lectin carrier. However, type 1 RIPs can still be internalized by fluid phase endocytosis.

Unlike lectins, **proteinase inhibitors** do not interact with the brush border of the small intestine, but are thought to exert their anti-nutrient effect by reducing the efficiency of digestion of dietary proteins in the gut. Trypsin inhibitors are powerful inducers of growth and continuous overstimulation of the pancreas (hypertrophy and hyperplasia) which may be one of the most harmful consequences of the long term consumption of diets containing trypsin inhibitors. Protease inhibitors are present in many common food components of human diet, including most legumes, cereal grains, potatoes and eggs. Trypsin inhibitors of soybeans are probably the best understood. They are normally inactivated by heat treatment, though commercially available edible soybean products retain 5–20 % of the trypsin inhibitory activity present in the original soybeans. Most informations of the nutritional effects of trypsin inhibitors come from experiments with animals. While some mammals are sensitive (rats, chicks) to PIs, others are not (pigs, dogs, calves). Because of this difference in species response, any prediction of the effect of trypsin inhibitors in humans is difficult.

In the case of lectins it is not easy to distinguish between **anti-nutritional and toxic effects**. Plant lectins overall effects – induced growth of the small intestine, shift of bacterial flora and interference with hormone secretion – may be “mild” and/or reversible compared to potential lethality arising from toxic proteins such as ricin. But there are some relationships between lectins and toxins: Lectins were designated as “toxic within brackets” by CARLINI & GROSSI-DE-SA (2002) following feeding experiments.

They can **“dramatically affect the entire digestive tract and its bacterial population, body metabolism and health”** (PUSZTAI & BARDOCH 1996).

“You often hear the generalisation, that lectins are toxic. There is some truth in it, but it it's not all lectins are toxic, it is the combination if you give them orally, that they must have some receptor binding ability in the gastrointestinal tract and it is also that they can get themselves injected into the cell and get trans-epithelially transported into the circulation” (EXPERT INTERVIEW 2006).

VASCONCELOS & OLIVEIRA (2004) describe symptoms of acute toxicity of lectins on humans: Nausea, bloating, vomiting and diarrhoea. In animal feeding studies the evident symptoms are loss of appetite, decreased body weight and eventually death. Yet they conclude that

“the mechanisms by which lectins mediate toxicity and the characteristic which dictate whether a lectin will be deleterious or not are not completely understood [...]. Actually the only reliable way of predicting the oral toxicity of lectins is by feeding the target animal on diets containing the purified lectin. It is also important to carry out these tests on several different animal species and not just on rats or mice. However it is not practical to test all lectins in-vivo. To establish a screening system, it is thus important to develop a clear picture of the lectins that lead them to be toxic to man and animals” (VASCONCELOS & OLIVEIRA 2004).

CARLINI & GROSSI-DE-SA (2002) claim that the applicability of most lectins known to have entomotoxic activity as plant-incorporated is largely restricted by their toxicity to mammals and/or non-target insects.

Generally regarding **safety evaluation of plant toxins** a lack of data is assumed by SPEIJERS (1995): **“If the situation of inherent plant toxins is compared with that of other categories of compounds such as food additives, pesticides and even environmental contaminants, it becomes obvious that for the safety evaluation of natural toxins essential gaps in the knowledge of toxicity exist.”**

Bacterial toxins generate their adverse effects either by acting on the target cell surface to irreparably damage the cell membrane, altering normal cellular signal transduction or exhibiting enzymatic activity in the cytoplasm. A very common way of uptake of bacterial toxins is via food. Considering ingestion, most food borne pathogens produce toxins that are involved in the disease process. Certain toxins such as botulinum neurotoxins are sufficient to cause symptoms after ingestion even in the absence of the producer organism, others are produced on the entrance of the gut without establishing infection (diarrhoeal toxin). According to the role of toxins specific types of mechanisms of pathogenicity can be generated:

Table 5: Mechanisms of pathogenicity.

Group	Mechanism
1	Enterotoxin or neurotoxin produced in foods (preformed)
2	Enterotoxin produced in the intestine without bacterial adherence to epithelial cell
3	Enterotoxin produced after bacterial adherence to epithelial cells, no bacterial invasion
4	Bacterial invasion localized to the epithelium and the intestinal immune system, with or without toxin production
5	Systemic infection

Source: ALOUF & FREER (1999)

Considering this classification, only group one manages to generate pathogenicity without an uptake of bacteria. Overall pathogenic symptoms may be: Abdominal pain, headache, bloody diarrhoea, nausea, vomiting and fever. Types of toxins involved in food poisoning are emetic toxins, neurotoxins and enterotoxins. Emetic toxins cause vomiting by binding specific receptors in the duodenum. Neurotoxins are proteins which act on the nervous system and enterotoxins are defined as proteins that are active in the intestine and cause diarrhoea. Enterotoxins are either disrupting the cell membrane causing cell death and cytotoxic enterotoxins, which enter the epithelial cell and cause diarrhoea without direct membrane disruption.

2 Protein Toxicity Assessment in GM Plants and Food

This Chapter provides a description and critical discussion of protein toxicity assessment in the context of GM plants and food/feed. It focuses on guidance documents and regulatory practice in the EU and the USA and also includes recommendations from international and national scientific bodies.

Section 2.1 introduces into the rationale and structure of protein toxicity assessment.

Section 2.2 to 2.9 describe each type of evidence required/provided in GM plant risk assessment: history of consumption/use, characteristics of the source organism, exposure, mode of action of the introduced protein, temperature stability, homology searches, in-vitro digestibility studies and animal studies. Section 2.8 discusses the nature of the test proteins used in the animal studies and also in the in-vitro digestibility studies. Section 2.10 focuses on the approach to exposure assessment, Section 2.11 on risk characterisation. A summary is provided in Section 2.12.

2.1 Rationale for protein toxicity assessment and overall approach

GM crops and food are, in accordance with recent guidance documents, individually assessed in a case-by-case approach (EFSA 2006a). Compared to checklist approaches in other regulatory contexts such as chemicals data requirements are less standardised. Consequently, the level of data requirements on protein toxicity assessment included in guidance on GM plant and food assessment varies a lot. Most detailed information is included in the EFSA guidance (EFSA 2006a).

Accordingly, the assessment approaches found in practice differ on the level of detail but follow a general pattern and typically include the elements described in the following (see also Figure 1).

Hazard identification

1. History of safe use/consumption (usually of the source organism).
2. Information on the mode of action and biochemical specificity.
3. Amino acid sequence comparison of the introduced protein to known toxins.
4. Acute toxicity studies in rodents (using in the vast majority of cases a microbially produced surrogate protein the equivalence of which to the plant-derived protein is established by a set of tests).
5. Temperature stability (to mimic food processing).

The history of safe use/consumption has been claimed for the vast majority of proteins introduced into GMPs which are authorised or pending for commercial application. Overlooking a broad range of risk assessment dossiers between 1995 and 2006, no homologies to known toxins were detected or reported as being biologically relevant. Acute toxicity studies are included in almost all dossiers, though, no toxicity studies seem to be available for a small number of authorised GMPs. Testing of temperature stability is also included in almost all dossiers.

Exposure assessment

1. Expression level in the (edible) plant tissue.
2. Description of food processing.
3. In-vitro digestibility studies: relevant for the bioavailability of the protein following ingestion.
4. Calculation of exposure to the protein based on food consumption databases and expression levels.

Risk characterisation

Risk characterisation is essentially limited to show a safety margin between worst case exposure and the minimum NOAEL (calculated on the basis of non-observed effects in acute toxicity tests) which typically exceeds an MoS of 100.

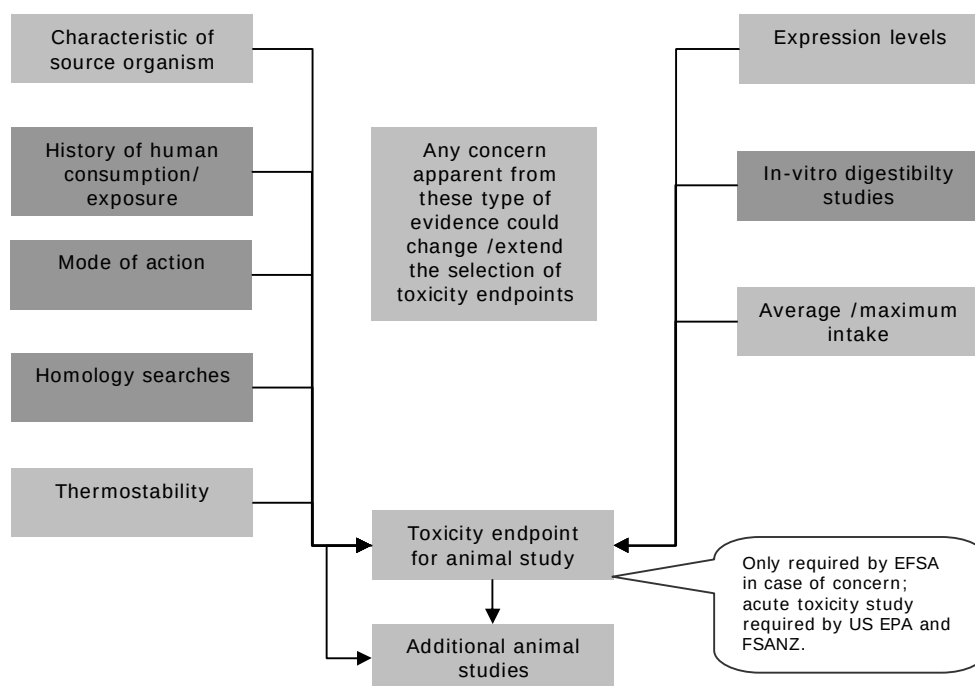


Figure 1: Overall flow chart of protein toxicity assessment in GMO risk assessment based on the approach of EFSA and US EPA.

2.2 History of safe use

The history of safe use/consumption is a key concept in the toxicity assessment of novel proteins thereby weighting experience in human exposure or consumption of the novel protein or the source organism containing the novel protein. According to the EFSA

Guidance (EFSA 2006a) in case of a well documented history of human consumption toxicity tests might not be required.

Experience with human exposure/consumption was described very vaguely and bluntly in risk assessment dossiers up to 2001 (SPÖK et al. 2003, 2004). Similar examples from more recent dossiers suggest that this practice continues.

- “The Cry1Ab protein has an insecticidal mode of action in the gut of certain in insects that is based on binding to specific receptors. The long history of safe use of this protein in microbial **Bt** products and its history of safe use in products derived from [...] further support its safety to humans and animals.”
- “There exists a long history of safety for the Cry1Ab protein”.
- “The CP4 EPSPS proteins belong to the class of EPSPS enzymes that are commonly found in a wide variety of food sources and have a history of safe use”.
- “There is a history of safe use of the CP4 EPSPS protein for human and animal consumption”.

The concept is also used in the overall risk characterisation to confirm safety:

- “The demonstrated safety of the proteins CP4 EPSPS and Cry1Ab, the history of safe use of (GM) soybean, which express the same CP4 EPSPS, the history of safe use of maize line (GM) maize since 1997, the safe history of use of the host organism as a common source of food and the determination of compositional equivalence, support the conclusion that there is no significant risk to humans involved in the handling, transportation, processing or consumption of [...] (GM) maize.”

The history of safe use approach is widely used in risk assessment, e.g. in the course of toxicity assessments of chemicals, food additives, and pesticides. A complete absence of a history of safe use – although not necessarily associated with a hazard – indicates that additional toxicological testing might be required. Conversely, a well documented history of safe use could lead to a waiving of test requirements. Hence, it serves to underpin conclusions about the overall safe application. It therefore also appeals to familiarity, but is has been seldom defined until now. Canadian guidelines provide one definition of “history of safe use” as **“significant human consumption of food, over several generations and in a large, genetically diverse population for which there exist adequate toxicological and allergenicity data to provide reasonable certainty that no harm will result from consumption of the food”** (HEALTH CANADA 2003).

A fundamental problem with this concept is that it usually refers to anecdotal evidence. Traditional food and food processing methods are very rarely ever subjected to systematic toxicity assessment because they are considered safe.⁵ Furthermore, there are normally no epidemiological studies available which would verify a history of safe use in a more scientifically sound way. This poses the question of weighting such type of evidence.

Applying this concept to complex structures such as proteins, micro-organisms, or plants also requires a more precise specification to what type of substance, fermentation or product the claim is referring to. Frequently the claim of safe use is rather referring to generic classes of proteins, microorganism and applications.

Thus, despite its frequent and widespread use the history of safe use is lacking scientific precision. Recently, an ILSI Task Force proposed a definition of the history of safe use

⁵ even though they may contain antinutrients, toxins or allergens

and described this approach based on regulatory recommendations (CONSTABLE et al. 2007) that if invoking this concept information should be provided on

- The period over which the food has been consumed.
- The way in which it has been prepared and used and at what intake levels.
- Composition and the results from animal studies and observations from human exposure.

2.3 Information on the source organism

Information on the source organism of the introduced protein represents a standard requirement in GMP risk assessment and is included in each risk assessment dossier.

Of particular importance is the precise taxonomic identification of the source organisms as this represents a prerequisite to reliably link existing information on or assumptions in the scientific literature about toxic, anti-nutritive or otherwise harmful properties to the source organism which is relevant when assessing the novel protein. Also of high relevance is any history of prior human exposure or use in food production (described in Section 2.2).

A closer look at the source organisms relevant for GM plants authorised or pending for commercial use in the USA or the EU reveals that the experience is so far limited to a small number of species and strains (see Table 6).

Table 6: Biological sources of novel proteins introduced in GMPs authorised or pending for commercialisation.

Protein	Donor
Proteins of microbial origin	
CP4 EPSPS, 2mEPSPS*, Nopaline synthase	<i>Agrobacterium</i> sp. strain CP4
Barnase, Barstar, RNase	<i>Bacillus amyloliquefaciens</i>
Cry2Ab, mCry3A, VIP3A	<i>Bacillus thuringiensis</i>
Cry1F	<i>Bacillus thuringiensis</i> sp. <i>aizawai</i>
Cry3Bb1	<i>Bacillus thuringiensis</i> sp. <i>kumamotoensis</i>
Cry1Ac	<i>Bacillus thuringiensis</i> sp. <i>kurstaki</i>
Cry3A	<i>Bacillus thuringiensis</i> sp. <i>tenebrionis</i>
Cry9C	<i>Bacillus thuringiensis</i> sp. <i>tolworthi</i> strain BTS02618A
Cry1Ab	<i>Bacillus thuringiensis</i> sp. <i>kurstaki</i> strain HD-1
Cry34Ab1, Cry35Ab1	<i>Bacillus thuringiensis</i> strain PS149B1
Cry2Ab2	<i>Bacillus thuringiensis</i> sp. <i>kurstaki</i>
APH4 (hygromycin-B-phosphotransferase), DAM, GUS, NPTII, PMI,	<i>Escherichia coli</i>
S-adenosylmethionine hydrolase	<i>Escherichia coli</i> bacteriophage T3
Nitrilase	<i>Klebsiella pneumoniae</i> sp. <i>ozanae</i>
GOXv247, GOX	<i>Ochrobactrum anthropi</i>
ACCd	<i>Pseudomonas chlororaphis</i>
PAT	<i>Streptomyces hygroscopicus</i>
PAT	<i>Streptomyces viridochromogenes</i> strain Tü494
Proteins of viral origin	
ALS	Chimera of 2 resistant AHAS genes
CMV CP	Cucumber mosaic cucumovirus
PRSV CP	Papaya ringspot potyvirus
PVY CP	Potato potyvirus Y
WMV2 CP	Watermelon mosaic potyvirus 2
ZYMV CP	Zucchini yellow mosaic potyvirus
Proteins of plant origin	
QPTase, ALS	<i>Nicotiana tabacum</i>
ALS	<i>Arabidopsis thaliana</i>
DFR, F3'5'H	<i>Petunia X hybrida</i>
GBSS	<i>Solanum tuberosum</i>
ACC	<i>Solanum lycopersicum</i>
Thioesterase	<i>Umbellularia californica</i>

*Double mutation of the EPSPS from *Agrobacterium* sp. strain CP4. Status 2005.

2.4 Mode of action

From a detailed knowledge on the biochemical mode of action of a protein some conclusions can be drawn about the expected physiological effects if it gets into contact with the human body. As proteins are frequently fulfilling roles as enzymes or in binding

to other proteins, DNA or other cell compounds the specificity of catalytic reactions and binding is of particular relevance.

The better the mode of action is understood, the less likely it is that the protein will cause some unexpected effect. An understanding of the mode of action is also important for developing a testing strategy.

Although not always explicitly referred to as “mode of action” the description of the biological function of a novel protein in both the source organism and the target plant is a standard requirement in guidance documents (e.g., CODEX ALIMENTARIUS COMMISSION 2003, EFSA 2006a) and is also routinely described in risk assessment dossiers.

For instance the plant EPSPS protein is the only known physiological targets of glyphosate in plants. Glyphosate does, however, not inactivate the **Agrobacterium** derived CP4-EPSPS (CP4 refers to the **Agrobacterium** strain) which, when expressed in plants, confers herbicide tolerance. Different to plants and microbes, mammals including humans are lacking the biosynthetic pathway for synthesising aromatic amino acids, thus do not have an EPSPS or analogous protein. From these insights no specific interaction with human biosynthetic pathways would be assumed.

Perhaps most research has been dedicated to the mode of action of Cry endotoxins. Following ingestion, the crystalline inclusions are dissolved and then the protoxin is activated by insect proteases. The active toxins bind to specific receptor sites and produce pores in the insect gut which is lethal to the insect. In addition, there may be other less-characterized insect control functions of these toxins such as avoidance of the toxin and feeding paralysis prior to completion of the full lethal pore-formation process (ARONSON & SHAI 2001).

Data from rodents do not indicate that there are adverse effects of microbial **B. thuringiensis** preparations (e.g. SCHNEIDER et al. 1998). This conclusion is supported by the negative results for purified Cry toxins tested on rodents in support of commercial use of transgenic plants.

Another important aspect is that binding of the active Cry toxin molecules to a receptor in the epithelial cells in the midgut of the target insect is an essential process in achieving toxicity (HOFFMANN et al. 1988). The ability to bind to specific receptor sites in the membrane is considered to be the most important reason for the host specificity of the Cry-toxins.

Mammals seem to be lacking receptors for binding of the Cry toxins (NOTEBOEN et al. 1995). Furthermore, the mode of action also appears to be insect specific due to the reliance on unique lepidopteran ATPases for potassium influx regulation and the insect midguts unique susceptibility to ionic stress (KNOWLES 1994). Even when Cry toxin binding site proteins are expressed in mammalian cells, the mammalian cells are unable to express them in a form that allows the toxins to bind to them (KEETON & BULLA 1997).

For these reasons effects on humans are considered to be very unlikely. The mode of action is usually described for the original or target organisms: in the examples described here these are **Agrobacterium** sp., **Bacillus thuringiensis** and its host insects as well as transformed plants. The mode of action of proteins transferred to other biological contexts might however still differ from its original context in an unpredictable way. This is illustrated by still ongoing discussion about possible biological effects of Cry toxins in humans, 12 years after the first GM plant containing a Cry1A(b) gene was authorised for market commercialisation. Using cultured bovine hepatocytes as a model system SHIMADA et al. (2003) described an increase of LDH after administration of Cry1A(b). In a subsequent work the authors found in

immunohistochemical and ligand blot analysis that Cry1A(b) toxin binds to actin, a cytoskeletal protein while at the same time there was no binding to membrane receptor or candidate proteins necessary for development of toxicity in susceptible insects (SHIMADA et al. 2006). While both studies generally confirmed the absence of acute toxicity on mammals they also highlighted that there are still open questions.

2.5 Temperature and pH stability

Plants are often processed prior consumption often involving shift in pH and/or high temperatures. Therefore, information on pH and temperature stability provides some indication of the structural and functional integrity of the protein in the final food.

Recent guidance documents usually include such information requirements (SCIENTIFIC INSTITUTE OF PUBLIC HEALTH AND BIOSAFETY COUNCIL 2003, CODEX ALIMENTARIUS COMMISSION 2003, EFSA 2006a). The EFSA and the Codex guidance explicitly propose to study the influence of processing on the novel protein. The EFSA guidance explicitly states in the section on toxicity assessment of the novel protein that “The stability of the plant expressed protein should be studied under processing and storage conditions and the expected treatment of the food/feed. The influences of temperature and pH changes should normally be examined and potential modification(s) of the proteins (e.g. denaturation) and/or production of stable protein fragments generated through such treatments should be characterised” (EFSA 2006a, p. 28).

Risk assessment dossiers occasionally include data on temperature stability of the novel protein, though endpoints are differing. Sometimes, the influence of temperature on immunorecognition was determined, sometimes the loss of enzymatic activity, of the loss of function or the degradation of the protein was investigated.

The rationale for the temperatures chosen is not always clear, for instance, whether and how they mirror particular processing steps; especially as heating steps could be very different, e.g. dry heating or wet heating.

Table 7: Data on temperature (and pH) stability of novel proteins available in risk assessment dossiers.

CP4 EPSPS	GOXv247
Cry1Ab	mCry3A
Cry1Ac	2mEPSPS*
Cry1F	PAT*
Cry34Ab1	PMI
Cry35Ab1	Thioesterase
Cry3Bb1	

**) Examples for heat stable proteins. Data on thermostability and pH stability (besides in-vitro digestibility studies) do provide relevant information in addition to influence of processing as food is sometimes processed in very different ways which cannot be easily covered by a few experiments.*

Stability testing will be especially true for novel proteins with which there is less experience in food contexts. Out of 12000 different enzymes included in the BRENDA database⁶ more than 800 are described to be stable at temperature 70 °C or lower and about 160 are stable at pH 2.5 or lower. This will also be relevant for novel proteins in plants dedicated for non-food purposes as these proteins might accidentally end up in the food chain following commingling or outcrossing. A first example of such a case is

⁶ <http://www.brenda-enzymes.info/>

maize 3277. In this case, a temperature stable amylase was deliberately introduced for technical purposes.

2.6 Bioinformatics

A frequently used method for inferring the biological function of a protein is by sequence similarity searching in protein and DNA sequence databases. In the course of GM plant and food risk assessment homology searches are used for three related purposes.

Typically, several protein databases (e.g. NCBI, GenPept, Swissprot, PIR, TrEMBL) containing publicly available protein sequences are consulted to identify the biological function and to reveal relationships between protein families.

First, homologies (and functional similarity) of the novel protein to proteins which have a history of safe human or animal consumption or to which there have been human exposure before are used to support the argument that the protein is not novel is unlikely to pose a threat to humans.

Second, homology to proteins with a well investigated physiological role and biochemical function could contribute in clarifying the mode of action and specificity.

Third, the newly introduced protein is checked against the amino acid sequence of proteins known or suspected to be toxic.

Although all three types of homology searches play a role in protein toxicity assessment, this section only deals with the third type. Usually, only with this type of homology comparisons detailed descriptions are included in the risk assessment dossiers.

The homology searches, in principle use the same overall approach that is used for searching matches with allergenic proteins. Different to allergenic properties there is no particular method proposed in guidance documents (e.g. more than 35 % identity over 80 consecutive amino acids, 100 % identity over 6–8 consecutive amino acids) which would help to identify potential toxins. Different to allergens toxins are usually not recognized by specific sequence motifs. Therefore comparisons are frequently made to databases of all available protein sequences.

Significant sequence similarity among proteins may imply that the proteins share the same secondary and tertiary structure and thus have close biological functions.

Homologous proteins have evolved from a common ancestor and thus share a common three-dimensional folding structure and in addition often similar active sites or binding domains. Homologous proteins frequently share the same biological function, but depending on the cell type or their subcellular localization they may have changed their functionality in entirely unpredictable way. The ability to characterize the biological properties of a protein based on its sequence alone stems almost exclusively from properties conserved through evolutionary time: the inference of homology (common ancestry) is the most powerful conclusion one can draw from a similarity search because homologous proteins share similar three-dimensional structures. Detecting homology may often help determining the function of new proteins and can be deduced with a high statistical confidence, given that the sequence similarity is significant.

Nevertheless, similarity does not necessarily imply homology⁷ and deducing biological function out of sequence similarity is not straight forward. Most sequence comparison algorithms are nowadays accompanied with statistical estimates which provide a measure of statistical significance of the observed similarity and thus help assessing the significance. A statistically significant similarity which helps to avoid false positives employs an expectation value (E-value) threshold < 0.01 (PEARSON 1995). Sufficient homology between two proteins is most commonly defined as 50 % similarity and 30 % identity of the total amino acid sequence. In recent GM risk assessment practice, the 35 % cut off proposed for identifying potential allergens is being used in all homology comparisons including those to known toxins (company information). This cut off is, however, considered to be too conservative and to deliver too many false positives.

This is also reflected in the GM risk assessment literature “the predictive value of the comparative sequence homology approach is still in an exploratory phase, and better understanding is needed about the relationship between type and degree of homology and [...] toxicity” (KUIPER & KLETER 2003, p. 281).

2.6.1 Homology comparisons in risk assessment dossiers

Homology comparisons of the introduced protein to known toxins are routinely conducted in toxicity assessment of GM plants and GM food and are included in essentially every risk assessment dossier. Very rarely searches are extended to specifically include anti-nutrients⁸ (see Supplement Volume). Homology comparisons are not routinely required/provided in other contexts of protein toxicity assessment such as food and feed enzymes.

This section describes and compares methodological details of a selected subset of fulltext homology studies including the following proteins: PAT, CP4-EPSPS, Cry1F, Cry3A, Cry34Ab1, Cry35Ab1, and PMI (see also Table 1 in the Confidential Supplement Volume).

2.6.1.1 Query sequence

In all studies sequence comparison was performed at the protein level, sometimes also the nucleotide sequence was used as query sequence.

The comparison of protein sequences has proven to be much more effective: the ability to identify distantly related sequences is dramatically reduced when DNA sequences are used. Though the evolutionary events occur at the DNA level, the main genetic pressure is on the amino acid sequence. Moreover, mutations at the DNA level do not necessarily change the encoded amino acid due to the redundancy of the genetic code and often result in conservative substitutions at the protein level. Such changes thus tend to have a minor impact on the protein's functionality. In addition, all non-coding regions are also considered when searching for similarity on the DNA level. Due to codon degeneracy and codon biases the use of protein sequences is certainly more significant.

2.6.1.2 Database used

The public databases GenPept including all translated DNA sequences from GenBank and EMBL, PIR and Swissprot, were most commonly searched with the respective query

⁷ Similarity between two proteins implicates that both proteins are descendants from a common ancestor (homology). Additionally similarity can also be found for two proteins if they share structural features (convergence to similar structure from independent origins). However, identity does not consider structural similarity or homology; identity simply refers to the overall percentage of identical amino acids across the whole protein length, and therefore must not imply homology.

⁸ It is possible but not clear from the dossiers that anti-nutrients are covered when conducting homology searches to entire databases.

(for basic information on protein database see Box 1). In some studies, specific subset databases were created by collecting all sequences retrieved from GenPept with the keyword “toxin”. As can be seen in Table 8 only a very small fraction of all entries in databases are associated with toxicity, though absolute number seems be very high. The majority of toxic proteins is still represented by bacterial proteins. Remarkably, an surprisingly high number of toxins can be found in the Protein DataBank (PDB), which also reflects the interest (mainly of the pharmaceutical industry) to find inhibitors by designing specific compounds based on the 3D structure (BERMAN et al. 2003). Other than with potential allergens, there is not comprehensive database on toxic proteins publicly available. The Tox-Prot program, the UniProt/SwissProt Toxin Annotation Program (<http://www.expasy.ch/sprot/tox-prot/>), aims to systematically annotate all proteins acting as toxins, but the some 2000 entries are restricted to venomous and poisonous animals.

Table 8: Number of sequence entries of proteins associated with toxicity.

Database		Version	All entries	Keyword			
				Toxic		Toxin	
				Sequences	%	Sequences	%
NCBI GenBank		Release 158.0	67218344	16177 (12174 bacterial)	0.024	59485 (49959 bacterial)	0.088
Uni-Prot	Swiss Prot	51.7	259034	118	0.045	2096	0.809
	TrEMBL	34.7	3826359	278	0.007	4652	0.121
PIR	iPro Class	3.26	4085393	6151	0.150	24433	0.598
PDB		15-03-2007	38368	61	0.159	1034	2.669

From the point of reviewers of risk assessment dossiers it might be preferred to not only include the detailed results of homology searches to known toxins (and allergens) in the dossiers. Either a full search should be documented or homology searches should be widened to include not only toxins but also proteins with anti-nutritive, hormonal, and pharmacological activities as well as proteins involved in pathogenicity. Entries can be carefully selected thereby avoiding false positive and false negative results compared to keyword generated database subsets. Ideally the database should also provide information about function, mode of action, post-translational modifications and 3D structural data.

Box 1: Amino acid sequence databases.

During the last three decades considerable effort has been made to create protein databases containing different types of structural information (e.g. primary amino acid sequence, secondary structure, 3D structure, protein family domains, consensus active sites etc.). Algorithms have been developed which compare sequences to detect evolutionary and thus structural and functional relations. Most databases are cross-referenced with each other in order to provide as much information as possible.

For homology searches, the use of a comprehensive database such as the **nr database** at the NCBI website (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>) is recommended (ALTSCHUL & LIPMAN 1999).

This database combines sequence data from the translated coding regions of DNA sequences in GenBank, EMBL and DDBJ as well as protein sequences submitted to PIR, SWISSPROT, PRF and PDB (BENSON et al. 2006).

Another comprehensive tool is the **UniProt database**, a central repository of protein sequences and function comprising of data contained in SwissProt, TrEMBL and PIR (BAIROCH et al. 2004). The SwissProt database maintained at the ExPASy center in Switzerland offers a lot of valuable biological information regarding protein function, domain structure and posttranslational modifications. This database is supplemented by the TrEMBL database, a computer-annotated collection of all the translations of EMBL nucleotide sequence entries not yet integrated in SwissProt. PIR integrates protein databases from PIR, JIPID and MIPS. Most interestingly this database (PIR-PSD) contains only amino acid sequences retrieved from protein sequences.

When searching for toxins, the **iProClass database** may be a useful tool, as it includes an assignment to protein families and functions (Wu et al. 2004). In addition the "PIR Pattern Match" enables to search for specific motifs. In general, searching for blocks, multiply aligned ungapped segments corresponding to the most highly conserved regions of proteins, may be helpful in identifying putative toxins.

It is worth mentioning that protein databases retrieved from DNA sequences such as the TrEMBL and GenBank Protein databases have a certain probability of delivering erroneous sequences, as the protein sequence may be deduced from sparsely characterized DNA sequences: the boundaries between exons and introns have to be correctly annotated to deduce the correct primary amino acid structure. In the case one intron is missed, the entire protein sequence gets affected.

2.6.1.3 Algorithm applied

Programs used to search databases differ in the core algorithm employed, thereby affecting both speed and sensitivity. Algorithms which have been particularly optimized for speed mainly use simplified assumptions in scoring sequence similarity and may therefore miss marginal, but still significant matches. Regarding the algorithm used to search for highly similar sequences, a trend to fast heuristic methods such as FASTA or BLAST can be observed in the studies analysed.⁹ Heuristic methods including FASTA and BLAST apply more assumptions to the sequence evolution, are much faster and more selective.

BLAST compares two sequences by seeking regions of similar sequences ("high scoring segment pairs", HSP) whose similarity score exceeds a certain threshold (T-value) (ALTSCHUL et al. 1990). The significance of each alignment of the query sequence with the database sequences helps in ranking the similarity hits. A higher T-value speeds up the comparison, but also leads to an increased probability of missing weak similarities.

Interpretation of the results of a sequence database search involves first evaluating the matches to determine whether they are significant and therefore imply homology. The most effective way is by considering the statistical scores (expectation values or E-values).

FASTA is another heuristic method performing fast sequence comparisons (PEARSON 2000). The algorithm starts by analyzing different regions and joining nearby high scoring regions. Finally, a dynamic programming is run around the best region, to obtain the "optimized score".

Two other parameters, the P- and E-values provide information on the significance of the similarity search. The E-value refers to the number of expected similarity hits for a given score T by pure chance. The probability that a similarity hit occurs by chance is

⁹ The choice of the algorithm is also depending on the company (company information).

given by the P-value. The lower the E-value (< 0.001) and the P-value are, the better is the chance that the sequences are truly homologous. Typically, comparisons between highly homologous proteins yield E values approaching zero, indicating the very low probability that such matches would occur randomly by chance. The probability that sequence similarities occurred by chance increases with higher E values. Alignments with E-values greater than 0.001 may still be homologous and can be analyzed for conserved functional motifs (PEARSON 2000).

In most initial searches the E-value was fixed at a rather high value ($E < 10$; $E < 1$).

Nevertheless, in 5 studies several randomly shuffled genes were created. Control BlastP searches were subsequently conducted using these shuffled versions as query sequence in place of the correct amino acid sequence. This procedure retained the overall amino acid composition but randomly rearranged the primary amino acid sequence within the protein. By running a similarity search with these randomly arranged proteins normally no significant matches should be found. However, the most significant match gives an overview above which E-value level the results obtained in a similarity search with the query sequence are significant and should be considered in further investigations: The range of E-values obtained for the modified sequences thus represent the background control incidence of random hits that could be expected based on the amino acid contained in the modified protein. E-values below this control range were considered to represent proteins of possibly significant amino acid homology. Nevertheless, all hits correspond to close homologs of the respective query sequence.

In none of the studies analysed more sensitive programs based on dynamic programming were used to find homologous proteins. Dynamic programming guarantees to find the best possible alignment by examining all possible alignments and is hence theoretically more sensitive in identifying distantly related proteins than both BLAST and FASTA. In addition, pattern-based, rather than similarity-based, comparison methods may be preferred when searching for functionally conserved non-homologous domains.

Furthermore, in none of the studies, the PSI-blast, a program capable of identifying distantly homologous proteins, was used. In addition motif databases, such as PROSITE, may be useful in searching for toxin motifs. However, motif identity in the absence of overall sequence similarity is not a reliable indicator of homology.

2.6.1.4 Parameters chosen

Matrix

Specifying an appropriate amino acid substitution matrix is central to protein comparison methods. Most commonly PAM and BLOSUM matrices are used in sequence comparisons. PAM matrices have an evolutionary model, whereas BLOSUM matrices are sampled from conserved regions at different average identity. Matrices representing greater evolutionary distances have larger PAM numbers or smaller BLOSUM numbers. Using these matrices will tend to find long, distantly related sequences while matrices with smaller PAM numbers or higher BLOSUM numbers will find shorter, more similar sequences.

The default matrices offered with the comparison algorithm (e.g. BLOSUM62 with BLAST, BLOSUM50 with FASTA, PAM250) are a safe choice and help in finding divergent sequences¹⁰.

¹⁰ Divergent proteins derive from the same ancestor, thus have the same overall structure, but do not share any statistically significant similarity, as the proteins have evolved in completely different directions over millions of years. Divergent proteins are often found in higher organisms, where cells have differentiated to highly specialized cell types.

Gap penalties

Gap scoring greatly affects search results, as gap scores change the sequence alignment and therefore the distribution of alignment scores. In general, selecting a large gap open penalty and a small gap extension penalty gives the most accurate results. All studies specifying the gap penalties have chosen rational values.

Filtering

The statistics for database searches assumes that unrelated sequences look essentially random with respect to each other. Long runs of a small number of different residues such as poly-alanine stretches may spuriously obtain extremely high match scores and can thus be filtered out.

In most studies the complexity filter has been turned off for the search, thus including sequences containing long repeated runs of a single residue or a pattern of residues in the search. This certainly helps in finding also distantly homologous proteins.

Word size

Lowering the “word size” parameter used for compiling words of query and database sequences, increases sensitivity. In the BLAST program the default value is 3 for proteins, in FASTA the word size (ktup) can be adjusted to either one or two.

2.6.1.5 Evaluation of hits

Sequence homology can be inferred from statistical significant similarity: unrelated sequences have similarity scores that are indistinguishable from random sequences (background; high similarity score). Statistically significant similarity (represented by a high similarity score) suggests that both sequences are related. Positive hits of the initial homology search are frequently evaluated by blasting its sequence back against public databases. This is described in one study only.

2.6.1.6 Results

The comparisons of the amino acid sequences to sequences of known toxins included in public databases demonstrated that none of the query protein sequences shares significant similarity with known toxins.

However, some of the homology searches were conducted more than 10 years ago. Sequence databases are constantly growing and especially during in recent years a tremendous increase in sequences associated with toxicity can be observed (see Table 9). A homology search conducted using recent databases may therefore yield different results.

Table 9: Entries of proteins associated with toxicity over years.

Fields Period	Substance name	Protein name	Keyword	Title	Text word
	Number of new entries				
Before 1993	0	0	0	0	0
1993-1994	46	61	579	476	3628
1995-1996	57	21	100	219	2651
1997-1998	77	28	95	276	3416
1999-2000	258	54	128	378	6371
2001-2002	173	159	328	854	28896
2003-2004	313	169	518	1124	29051
2005-2006	253	398	552	2235	60740
Total	1177	890	2300	5562	134753

The NCBI protein database was searched using the keywords "toxi" with the publication date set to the respective years.*

All studies analysed used local alignment tools, such as BLAST, which are suitable for reasonably similar sequences. However distant evolutionary related proteins are missed by this kind of search. Superfamily relationships can only be detected with more sophisticated sequence methods.

Inferring function from homologous sequences is a process fraught with difficulty. If the score is extremely good and the alignment can be found over the entire proteins, there is a good chance that they will share the same or a related function. However, deriving protein function is risky as functions do diverge.

Moreover, a significant fraction of functional annotations in databases are wrong. If only a portion of the proteins align, two proteins may share a domain which only contributes one aspect of the overall function.

Database search methods are also limited because most homologous sequences have diverged too far to be detected by pair wise sequence comparison methods. Thus, failure to find a significant match does not necessarily indicate that no homologs exist in the database. In such cases more sophisticated methods must be applied including iterative search programs such as the profile-based PSI-BLAST program though, they can easily lead to false positives by diverging from the original query sequence.

2.6.2 General remarks

Significant sequence similarity needs careful interpretation. High homology on the amino acid level simply suggests that the proteins **could** share a similar function. Homologies found in or near the active site have certainly more significance for the protein function than those in sites that are more distant; however, a protein may also act as a toxin via a binding event. In cases of **Bt** protoxins, which have to be proteolytically processed to yield the active toxin, both sequences, of the protoxin and the active toxin, have to be considered in similarity searches. There are also limitations in the ability of such comparisons to detect regions composed of different structural parts capable of interacting

Conversely, absence of significant sequence homology needs confirmations from other kind of studies. Given the dynamic growth of sequence entries in databases associated with toxicity periodical update might also be required. This is especially relevant for the renewal of market application or if the same protein is expressed in other GM plants.

Moreover, without considering the three-dimensional structure and exact arrangement of the amino acid residues, no clear conclusion can be made: about 20 % of the amino acids in a protein sequence determine the overall tertiary fold. As structures change more slowly than amino acid sequences and are hence more highly conserved than protein sequences, structure comparison may be favourable in finding many homologous proteins than sequence comparison. On the other hand, sequences are much easier to compare – not only due to the fact that sequence databases are much more comprehensive compared to structure databases e.g. the PDB database.

The development of more detailed guidance on homology comparisons have so far focused on allergenicity assessment whereas nothing was done on toxicity assessment.

As described above there is some variation in search parameters and databases for homology searches and an overall tendency to apply fast but less sensitive methods. The variations detected in our comparative analysis may not substantially influence the results. Nevertheless, in the absence of detailed guidance the variations might be larger than those identified in our analysis.

2.7 Nature of test substance

In case of GMPs almost all test protein subjected to toxicity relevant studies is produced from microorganisms, mainly *E. coli* and occasionally other bacteria (*Pseudomonas fluorescens*, *Bacillus* spp.). Purification of the plant expressed protein is technically feasible but would be much more costly especially in cases where protein concentration in plant tissue is the range of nanograms. This approach is generally accepted by regulators (e. g. FIFRA-SAP 2000, CODEX 2003, EVANS 2004, EFSA 2006a).

In cases the same gene is expressed in plants and microbes, the resulting protein might be slightly different. Differences could occur on the level of DNA sequence during transformation and in RNA splicing eventually resulting in an altered amino acid sequence as well as protein folding. Posttranslational processing including type and degree of proteolytic processing, glycosylation, acetylation, phosphorylation, methylation might also differ between plants and microbes. Any structural changes between the plant and the test protein may not necessarily but could be safety relevant as these changes could alter functional properties and thereby influence properties relevant to toxicity. This is generally acknowledged in the scientific literature and in guidance documents (e.g.

JONAS et al. 1996, KUIPER & KLETER 2003, EVANS 2004). For instance, proteins are frequently glycosylated in plants but are usually not glycosylated in bacteria.¹¹ Even proteins from microbial sources which would not be glycosylated in their original source organisms might be accidentally recognised by the plant post translational machinery.

There is at least one case of glycosylation of a *Bt* toxin in case of heterologous expression in insect cells using a baculoviruses system (MARTENS et al. 1995). In other contexts of protein toxicity assessment such as food or feed enzymes proteins are produced from the same microbes using the manufacturing conditions identical to commercial production. Therefore, equivalence of the microbial test protein and the plant expressed protein has to be supported by sufficient evidence. The robustness of equivalence studies is important, as the microbial protein is applied in animal toxicity studies and in-vitro digestibility studies.

¹¹ An exception to this are plant chloroplast proteins which are not glycosylated. Therefore, novel genes targeting plant chloroplasts would also not be glycosylated.

The Codex Alimentarius Commission proposes that the microbial protein should be “structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA plant” (CODEX ALIMENTARIUS COMMISSION 2002). Other guidance documents provide more detailed suggestions on the kind of data that should be provided to suggest protein equivalence (see Table 1 in the Supplement Volume). While the list of data requirements is very similar in all guidance documents it is usually neither understood as a comprehensive list nor as a standard data set which would be required in each case. All documents compare molecular weight, amino acid sequence, post translational modification, and immunological reactivity. Isoelectric point, and enzymatic activity is not considered by the EuropaBio and the US EPA. Conversely, functional analysis is not considered by the EC’s scientific committees and EFSA. More recently, mass spectroscopy analyses have been submitted (e. g. Cry1F, Cry3Bb1; data not shown).

Comparing ten different equivalence studies in risk assessment dossiers reveals some heterogeneity in evidence provided (see Table 2 in the Supplement Volume). No type of evidence is provided in each dossier. Most consistently, SDS-PAGE is used to compare molecular weights and Western Blot Analysis is applied for immunological reactivity. Less consistently but still in the majority of cases N-terminal sequencing is applied to investigate possible modification in protein structure and carbohydrate staining is used to identify any glycosylation. In some cases ELISA is applied in addition to Western Blot analysis, enzyme assays are conducted and functional properties are compared. In case of Cry proteins insect bioassays are used to compare functional properties. Rarely, additional experiments are conducted (proteolysis). The isoelectric point was not determined in any of the dossiers investigated.

Different sets of equivalence data are provided even in case the same or similar proteins are used. For instance with Cry1Ab ELISA is applied by one applicant but not by another one. In case of CP4-EPSPS and mEPSPS there is considerable variation in the data set including carbohydrate staining, ELISA, enzymatic assay. In case of a fodder beet intended to be used in feed only no equivalence data were provided at all.

The degree of information provided varied between studies ranging from full text reports describing experiments and results in great detail to only briefly mentioning results or referring to internal reports not included in the dossier.

The practice of using test proteins from different sources compared to the commercial products and the methods applied to compare the plant-derived and microbial protein have been criticised by a number of authors (GURIAN-SHERMAN 2003b, FREESE & SCHUBERT 2004, SPÖK et al. 2004, FIFRA-SAP 2000) as the equivalence data set cannot exclude differences in protein folding and in posttranslational processing. Conversely, contaminants in microbial extracts might lead to false positive results (FIFRA-SAP 2000).

The plant-expressed genes are frequently modified in their nucleotide sequence in order to optimise plant gene expression. Sometimes also the amino acids sequence is thereby slightly modified (e. g. GAO et al. 2004). According to a report by a Panel of US Academy of Sciences it is therefore “always a question of whether the final amino acid sequences are identical (that is, cloning artifacts could lead to some amino acid substitutions). The sequencing of some of the terminal and internal amino acids cannot itself prove identity; however, combined with results of other tests, the finding that these sequences were identical offers additional support of equivalence.” (NRC 2000, p 117).

In case of **Bt** proteins the NRC Committee also criticised the cross-reactivity of antibodies used, and the fact that for the tests on functional equivalence, two closely related species are being used (ibid.).

As a result absence of any harm from animal toxicity studies is still associated with uncertainty if the microbial protein actually reflects the real world situation. This is particularly true in the light of the heterogeneity of the data set as shown in this report.

The NRC Committee concluded that “tests should be conducted whenever possible using the protein as it is expressed in the plant”(ibid, p 14). The committee also recommends that “clear, scientifically justifiable criteria for establishing biochemical and functional equivalency when registrants request permission to test non plant-expressed proteins in lieu of plant expressed proteins” should be provided.

In order to take a closer look at the feasibility argument we calculated the total amount of test protein needed for various types of animal studies, based on typical expression rates, and estimated the minimum amount of plant biomass needed. The results are shown in Table 10.

Expression rates of novel proteins in presently used GM plants cover a broad range from a few nanograms expressed protein per gram fresh weight plant biomass (e. g Cry1Ab) to several hundreds micrograms per gram fresh weight (e. g. Cry3Bb1, CP4 EPSPS). Expression levels of the same gene in different plant species and events as well as expression in different plant tissues also show high variability. For the former group even the amount of test protein needed for single-dose acute toxicity studies in mice would be very difficult in case of expression rates below 1 microgram per gram fresh weight plant biomass. For example, about 1200 kg of plant biomass would be required for maize Bt11. In contrast, for the latter group purification from plant biomass would perhaps be feasible to provide sufficient test protein for 28-day subacute studies (the type of study proposed in the EFSA guidance document).¹² In rare cases expression even reaches milligram levels.

The amounts necessary for subchronic studies would, however, be beyond what could possible be achieved based on expression levels in presently used GM crops.

¹² GM events of the latter groups include Cotton MON531 x MON1445, Maize MON88017, Maize MON863 x NK603, Sugar beet H7-1, Roundup Ready Cotton MON-15985-7, Maize MON863 and MON863 x MON810, Cotton MON15985 and MON15985 x MON1445.

Table 10: Amount of test protein required in toxicity tests. bw = body weight

Toxicity endpoint	Trial design ⁷	Species	Amount of test substance ²	Minimum plant biomass [kg] required depending on expression level of the novel protein [$\mu\text{g/g fw}^1$]		
				1	100	1000
Acute toxicity	5 male mice, single dose, 2500 mg/kg bw ⁵	Mouse	Ca. 0.45 g	450	4.5	0.45
28-day repeated dose toxicity	5/dose/sex, 2, 20, 200 mg/kg bw/day ⁶	Mouse ³	Ca. 2 g	2000	20	2
		Rat ⁴	Ca. 19 g	19000	190	19
90-day subchronic toxicity studies	10–20/dose/sex, typically 400, 1200, 5000 mg/kg bw/day	Mouse	Ca. 630 g?	n. c.	6300	n. c.

¹ Not considering losses during purification.

² Assuming pure protein.

³ Assuming an average bw for males of 36 g, for females 26 g.

⁴ Assuming an average bw for male of 130 g, for females 170 g.

⁵ Max. 5000 mg/kg bw: 3–5/dose/sex.

⁶ Max. 1000 mg/kg bw/day.

⁷ Trial design modelled according to examples described for protein toxicity studies in the context of GM plants or Novel Foods.

The hundreds of kilograms of biomass which have to be processed for subacute tests still might require substantial efforts.

Efforts and costs have to be seen against the backdrop of microbial fermentation. Even if test protein is produced from microbes, according to EVANS (2004) 100 to 1000 l fermentation scale is required to produce gram amounts. For instance, the yield of NptII was described to be in the range of 30 g from two fermentation batches in 150 l tanks. In case of CP4 EPSPS purification from *E.coli* in 60 l fermenter resulted in 260 mg of protein, in case of the PAT protein less than 1 mg resulted from 50 l scale. GUSTAFSON et al. (1997) described the production of 50 g of the 68 kDa protein of *B. thuringiensis* (Cry3A) from *E. coli* at the 1000 l scale. 31 and 37 g of 98 % pure Cry34Ab1 and Cry35Ab1, respectively, could be obtained using the applicants own proprietary high-yield production system in *Pseudomonas fluorescens* on 75 l scale (HUANG et al. 2007). Surrogate proteins might also fail to meet equivalence criteria in the first place, thus resulting in the need to explore several expression systems before scaling up production. Therefore, substantial efforts and costs may also arise from producing the test protein from microbes if more than acute toxicity tests are intended.

For subchronic and in particular chronic toxicity tests which have been demanded by some critics substantial improvements of yield by optimising protein expression and/or downstream purification and even scale-up of production might be required.

Unfortunately, no figures are available which would allow for comparison of costs.

On the other hand achieving equivalence status for microbial proteins might become more difficult if considering plant proteins. The vast majority of the proteins presently expressed in GMPs are of bacterial origin and are not expected to be glycosylated in their original hosts. They might be glycosylated in-planta only in case of 'accidental'

presence of glycosylation sequence motifs. In contrast, glycosylation of proteins from plant or animal sources is much more widespread.

So far, there is limited experience in risk assessment for market stage in assessing plant derived proteins expressed in other plants. Overlooking GM crops authorised in the EU, the USA and elsewhere, there have, so far, been only a few cases of plant proteins being expressed in other plants, e. g., flax CDC-FL001-2, canola 23-198 and 23-18-17, a few carnations and one tobacco variety (see Supplement Volume). As far as it can be revealed from publicly available documents, even in these cases the test protein was of microbial origin.

The carnation varieties and the tobacco are not intended to be used as food or feed and the canola contains a plastid protein from California laurel. Flax is also of little importance as a food crop. Plastid proteins are not known to be glycosylated. There is however a pipeline of at least 1400 different genes expressed in plants¹³ including a broad range of non-microbial and non-viral genes. Such genes are clearly targeted in the production of industrial substances in GMP (plant molecular farming, third generation traits) and play an increasing role in second generation traits (modifications of consumer-relevant plant/food properties). These proteins will more likely require plant specific post translational processing, thus, the use of surrogate proteins from microbial origin might no longer be possible. In case of plant molecular farming concentration of proteins in plant tissue can be expected to be very high. In case of complex traits involving the modification of entire pathways several genes might be expressed at low levels, each of them might needed to be produced in test substance quantities. In case of low-level expression the concept of equivalence might therefore still play a role.

Given the achievements in using plants as protein producing factories, test proteins might also be produced from plants and purified e. g. using affinity chromatography¹⁴. Viral transient expression systems established for tobacco allow for rapidly producing gram amounts and yields of up to 5 g/kg biomass (reviewed in SPÖK & KARNER, forthcoming). Still, as long as rapid transient expression systems are not available for a broad range of food crops, equivalence between the tobacco derived protein and the protein from the target plant might needed to be established.

Production of the novel protein from the original GM plant would definitely improve the relevance of any conclusions based on tests on the isolated protein. Nevertheless, a plant-derived protein does not automatically yield a protein identical to the in-vivo one, as the purification process can also have an impact on structural and functional properties. This is a general limitation which is almost impossible to avoid regardless of the production source but should be kept in mind when designing the purification step.

2.8 In-vitro digestibility tests

Exposure is deemed to be of less concern if a protein is shown to be rapidly degraded in the human or animal gastro-intestinal (GI) tract (e. g. THOMAS et al. 2004). This is relevant for both, toxicity assessment and allergenicity assessment.

GMP risk assessment dossiers most frequently include in-vitro assays applying simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) as indicators of gastric digestion instead of more complex and more costly in-vivo studies. Almost all heterologous proteins produced in GMPs so far, have been subjected to such in-vitro

¹³ 1400 different genes are included in the APHIS field trial database only (<http://www.isb.vt.edu/cfdocs/isblists2.cfm?opt=16>).

¹⁴ This method is also well established for purifying lectins and enzymes.

digestibility (IVD) studies (see Supplement Volume). IVD studies are also part of risk assessment in rare cases of novel food proteins (e. g. LEWIS 2006) but are normally not applied in other contexts of protein toxicity assessments such as food and feed enzymes.

IVD in guidance documents

Risk assessment guidance documents have provided contradictory advice on digestibility testing (see Table 1 and Box 2). The Scientific Committee on Plants (SCP) initially discouraged in-vitro tests and recommended in-vivo tests (SCP 1998). The Scientific Steering Committee (SSC) and the OECD pointed out their limited validity (SSC 2000, OECD 2000) while more recent guidance documents are bluntly encouraging IVD studies. The SCIENTIFIC INSTITUTE OF PUBLIC HEALTH AND BIOSAFETY COUNCIL (2003) considered IVD studies to be a trigger for further studies rather than an endpoint, other consider this type of studies to be a toxicity endpoint in itself when used in a weight of evidence approach (HÉROUET et al. 2005).

Table 11: Diverging opinions on in-vitro digestibility testing in guidance documents for risk assessment of GM plant and GM food.

Source	Testing requirements
SCP (1998)	Discouraged, in-vivo tests suggested
EPA (2000)	Encouraged for Bt proteins only
SSC (2000)	Limited validity (best case situation only)
OECD (2000)	"May not always provide clear evidence"
SCIENTIFIC INSTITUTE OF PUBLIC HEALTH AND BIOSAFETY COUNCIL (2003)	Encouraged (b); ex vivo gastric fluid test (e.g. pig, cattle) or in-vivo models on a case-by-case basis
EFSA (2006a) (a)	Encouraged
CODEX ALIMENTARIUS COMMISSION (2004)	Encouraged (c)
EU practice in GMP Dossiers (1995-2006)	Conducted for virtually all proteins, mainly SGF, less frequently also SIF

a) Also encouraged by EFSA (2006) for protein safety assessment in case of GMM.

b) Considered to be a trigger for further tests rather than a toxicity endpoint.

c) Only mentioned in the context of allergenicity assessment.

Source: Spök et al. 2004, extended

Box 2: Different views on the use of in-vitro digestibility studies in guidance documents.

"Evidence of degradation of the introduced gene products should be based on data obtained in-vivo by feeding the GM plant material or its derived products to the intended target animal. [...] The use of in-vitro simulation of gastric and intestinal digestion of the gene product should be considered supplementary to in-vivo experiments designed to measure the survival of the gene products when fed to animals as an integral part of the GM plant. Isolated proteins are known which are fully degraded in the simulated gastric system but survive gut passage intact when fed as part of a normal diet" (SCP 1998).

"There are arguments that the currently used tests of gastric and intestinal protein resistance to gastric and intestinal hydrolysis represent a 'best case' situation and do not reflect the digestive capacity of the very young and those with pancreatic and severe gastric disorders. The fact that a protein is digested does not preclude for some eventual pharmacological/toxicological properties of derived peptides. Such properties have been observed for conventional foods and thus should be anticipated in all foods, whether they are derived from GM protein or from non-GM protein" (SSC 2000).

"[...] the simulated gastric fluid (SGF) test, an artificial system for testing proteins' digestibility, does not mimic exactly the physiological conditions in the digestive tract. Such testing may not always provide clear evidence of the possible toxic or allergenic potential of peptides formed as breakdown products in the test system" (OECD 2000b, p. 14).

"Tests normally used to assess toxicity include in-vitro digestibility of the protein which is used to compare the properties of the novel gene product to the characteristics of known proteins. This test is not intended to detect any potential toxicity in the very young, the elderly, and that segment of the population which is unable to produce stomach acid. Test methods should be designed to evaluate potential risks for those subjects when the gene product is similar to a chemical that is implicated in having unique toxicity when not digested" (OECD 2000b, pp. 26–27).

"Data concerning the resistance of the novel protein to proteolytic enzymes (e. g. pepsin) should be obtained, e. g. by in-vitro investigations using appropriate and validated tests" (SSC 2003, p. 19).

"An in-vitro digestibility assay in simulated gastric and/or intestinal fluids is required. It is important to note that resistance to in-vitro digestion is not a toxicity endpoint by itself, but simply an indication that the protein warrants closer examination and perhaps different types of testing. On a case-by-case basis, also an ex-vivo gastric fluid test (e. g. pig, cattle, dog) or in-vivo models may be required" (DUTCH BIOSAFETY COUNCIL 2003, p. 11).

Methodological design

IVD have been criticised by some authors with respect to their relevance and methodological design (CSPI 2003, GURIAN-SHERMAN 2003a, b, SPÖK et al. 2004, VALENTA & SPÖK, forthcoming) A in-depth comparative analysis of IVD studies described in risk assessment dossiers and the scientific literature reveals differences in the experimental design (see Tables 2 and 3 in the Confidential Supplement Volume).

This methodological variability has to be seen against the backdrop of a recent discussion in the scientific literature on methodological aspects and on the relevance of IVD for the in-vivo situation. In general these papers focus on the role of IVD for allergenicity assessment but some aspects are equally relevant for toxicity assessment.

Several authors discussed that the choice of parameters including enzyme/test protein ratio, pepsin purity, target protein purity, protein staining method, and buffer pH could considerably influence the outcome (FU et al. 2002, BANNON et al. 2003, THOMAS et al. 2004, FIFRA-SAP 2005, HERMAN et al. 2006).

From the comparative analysis of IVD studies in risk assessment dossiers differences were in fact revealed in most parameters.

pH conditions (SGF only): Although variability in pH is little (pH 1.2 or 2.0), it might still be relevant. THOMAS et al. (2004) for instance showed in a SGF ring trial involving 9 laboratories that consistence across laboratories drops to 77 % at pH 2.0 compared to pH 1.2 (91 % agreement).

Method to monitor degradation: some designs use SDS-PAGE staining only, others include Western blotting. FIFRA-SAP (2005) also reported the use of HPLC. All these

methods differ in various respects, e. g. in their sensitivity and the ability to track breakdown products (FIFRA-SAP 2005).

Staining of the SDS-PAGE: The sensitivity of the staining method obviously influences the outcome (silver, colloidal gold, commassie blue, bromphenol blue).

Ratio of enzyme to test protein: The most striking differences were found in the ratio of enzyme to test protein. In SGF assays using pepsin the ratio varied about 5×10^3 fold, between 1:2 to 2500:1. Fu et al. (2002) describe even larger ranges of 1:250 to 5000:1.¹⁵ Using the example of the major egg allergen ovalbumin they showed that a too high ratio of enzyme/test protein could deliver false negative results. In contrast ASTWOOD & FUCHS (1996a, b) recommended a ration of 13:1, a recent FAO/WHO consultation about 6.5:1 (FAO/WHO 2001). The application of a too high enzyme/test protein ratio has also been critically raised in the context of GM crop and food authorisations pointing out the possibility of false negative results (GURIAN-SHERMAN 2003a, b, CSPI 2003).

The literature discussion did however only consider weight ratios. Given the huge variations in enzymatic activity of the pepsin preparations between 10 and 4500 U/mg this leads to a skewed picture. A more realistic range can be calculated if ratios of enzyme activity and amount test protein are calculated. Nevertheless, variability is even greater and up to 105fold, from 0.014 to 4000 U/ μ g test protein (see Table 2 in the Confidential Supplement Volume).

Whether the variability actually translates into different results of degradability certainly depends on the fact whether excess enzyme concentration is applied the details of which are still a matter of debate (FIFRA-SAP 2005, HERMAN et al. 2007).

A similar picture emerges from comparing SIF studies (see Table 3 in the Confidential Supplement Volume). Ranges of enzyme to test protein vary between 1:2 and 2500:1. Although the enzyme activity of the pancreatin preparations used does not allow for a more accurate calculation of enzyme activity:protease ratios, it can be assumed that these would lead to similar ranges.¹⁶ Such differences might be one reason for the differences in the assessments, for instance CP4-EPSPS is considered as "resistant to SIF digestion" in one study and "more than 50 % degraded after 10 minutes" in another study. Two of the IVD studies on CP4-EPSPS analysed resulted in similar degradation rates while differing 400fold in pancreatin:test protein ratio (see Table 3 in the Confidential Supplement Volume).

A related aspect which could lead to variability are the differences in the interpretation of experimental results: some have considered proteins non-stable when degraded within 30 seconds, others within 30 minutes (see also Fu et al. 2002, BANNON et al. 2003).

Both, SGF and SIF, have to be considered independently as there may not be a correlation between these assays. For instance, Cry proteins are easily degradable in SGF but more resistant in SIF, while most other heterologous proteins that have been tested in both systems were quickly degraded in both systems.

Given these methodological inconsistencies and the variations in results some authors have suggested standardized protocols. THOMAS et al. (2004) reached a fairly high level of consistence in a ring study involving 9 laboratories using standardises SGF at pH 1.2 or 2.0 and a ratio of 10 U pepsin per μ g test protein. A recent FIFRA-SAP Consultation

¹⁵ Studies on proteolytic digestion on allergenicity and nutritional studies used comparatively low ratios ranging from 0.1 to 0.001. Studies conducted for the purpose of safety assessment have, however, used higher ratios ranging from 25 to 5000 (reviewed in Fu et al. 2002, 2003, Bannon et al. 2003).

¹⁶ Peptidase activity should be 25 U peptidase/mg pancreatin (USP 22) or minimum 25 U (USP 23, USP 24).

sparked by a dispute on IVD-studies in the context of Cry34Ab1 came up with a number of recommendations, including a standard assay to be established to define pepsin activity and preferred using a fixed amount of enzyme units in digestion studies. The reasons provided for the latter are “a) allow replication of the study; b) account for ‘inactive enzyme molecules’; c) provide better control of ‘enzyme activity’ in relation to substrate concentration; and d) eliminate effects of batch-to-batch variations in enzyme preparations (with respect to enzyme activity) on experimental results” (FIFRA-SAP 2005: 14). HERMAN et al. (2006) suggested a kinetic approach to analysis of degradation by quantitative densitometry to overcome the problem of different interpretations. Others have found reverse phase HPLC more reproducible than scanned densitometry readings (LEWIS 2006). Given the results of the calculations above the amount of pepsin used should be based on enzyme activity rather than weight. The latter has also been suggested in a most recent paper by MORENO (2007).

Relevance for the in-vivo situation

A second aspect is how well the IVD studies reflect protein stability in-vivo. Some authors have acknowledged that the SGF assay may not fully simulate in-vivo gastric digestion which is considered to be variable across individuals and within individuals over time (e. g. BANNON et al. 2002; BURNETT et al. 2002; CHIKWAMBA et al. 2003; MENDELSON et al. 2003).

For instance the buffering effect of foods can influence the pH found in the stomach of healthy adults. pH can increase up to values around 3 after food intake (FORDTRAN & LOCKLEAR 1966) and can be as low as 1.5 when individual fasts and the stomach is empty (FALLINGBORG et al. 1989).

MORENO (2007) describes the influence of food matrix and protein hydration status.

Proteinase inhibitors present in some plants may reduce proteolytic activity in the stomach.¹⁷ JENSEN-JAROLIM & UNTERSMEYR (2005) pointed to the fact that elevated stomach pH is likely to result in a decrease of proteolytic activity, e. g. in early childhood, in elderly, or in chronic atrophic gastritis. Moreover, there are a number of pathologies, like gastritis or ulcer, where acid neutralization or inhibition is an important therapeutic goal. Acid-neutralization is state of the art during surgical care, corticosteroid or analgetic treatment. Anti-acids, H2-receptor blockers and proton pump inhibitors are increasingly consumed without prescriptions due to liberalization of the market by over-the-counter sale. Recent animal and clinical studies have confirmed that these drugs support IgE induction and sensitization to food allergens (UNTERSMEYR et al. 2003, 2005). Proteins can exhibit sensitizing and allergenic properties also in case of partial degradation – as long as there are intact epitopes. Toxic action in contrast to immunogenic action might require a functionally intact protein, thus the relevance of the latter studies are not entirely clear.

The limited relevance of IVD studies for the in-vivo context has been acknowledged in the scientific literature (BANNON et al. 2003, HERMAN et al. 2003) in the course an US EPA review (MENDELSON et al. 2003):

“The digestibility test is not intended to provide information on the toxicity of the protein or imply that similar breakdown will happen in all human digestive systems.” (MENDELSON et al. 2003, p. 1005).¹⁸

¹⁷ This and some other insights into the fate and bioavailability of proteins come from studies exploring oral drug delivery of biopharmaceuticals (e. g. Kong et al. 2001).

¹⁸ However, this qualification did so far not change the EPA's position that acute toxicity tests and in-vitro digestibility tests would provide a sufficient database to conclude the absence of toxic properties for Bt proteins.

Another issue is – even if a protein is degraded in the GI - the remaining fragments might excerpt biological function. This is a particular issue in allergenicity assessment where protein fragments can still have immunologically relevant epitopes (reviewed in THOMAS et al. 2004). The role of such breakdown products for toxicity assessment has so far not been considered.

MORENO (2007) therefore suggested dynamic in-vitro models to better simulate the nature of the human digestive system. Multi-phase model could mimic the passage of food into stomach (phase 1) and then into the gut (phase 2). Dynamic models could also consider transit and peristalsis.

2.8.1 Evidence from animal studies

Complementary but as of yet contradictory evidence comes from in-vivo studies with animals fed on GM plants. The digestive fate of novel proteins from GM plants has been investigated by a number of authors. HARRISON et al. (1996), ASH et al. (2003) and JENNINGS et al. (2003) show that novel proteins from GM soybeans were degraded in the GI tracts. PHIPPS et al. (2005) failed to detect PAT in the milk of cattle fed on GM maize.

RICHARDS et al. (2003) reported that with rats fed 1 mg per day of the green fluorescent protein (GFP) the protein was evident only in small intestinal contents of rats orally administered GFP one hour before killing. ELISA indicated the presence of not detected the small and large intestinal contents, faeces, liver homogenates or serum of rats chronically fed diets with the GM rape.

In contrast, CryA1b was detected in stomach, duodenal, ileal, cecal, and rectal contents of pigs fed diets containing 600 g Bt11 maize/kg (CHOWDHURY et al. 2003b) and the coefficient digestibility of Cry1Ab was estimated to be 0.92. Other authors reported the detection of transgenic CryA1b in the GI tract of ruminants and chicken including the faeces (CHOWDHURY et al. 2003a, EINSPANIER et al. 2004, LUTZ et al. 2005, JACOBS et al. 2005, WIEDEMANN et al. 2006).

PALKA-SANTANI et al. (2003), based on feeding of glutathione-S-transferase to mice, did not find degradation of the protein in stomach/small intestinal contents, and trace amounts in kidney extracts, 30 minutes or more after feeding.

Basically, all studies use ELISA and polyclonal antibodies for detecting the protein. This methodology, however, needs careful interpretation in order to discriminate between the full size and fragmented protein (WIEDEMANN et al. 2006). LUTZ et al. (2005) recommended that samples tested positive by ELISA should be reassessed by another technique (e. g. fragmented immunoactive epitopes).

The evidence from cattle and chicken might only be considered relevant for GM feed whereas the studies on swine are of particular relevance for human given the similarities in the GI system. The latter type of studies provides in fact evidence that questions the relevance of IVD studies.

2.8.2 Absorption of polypeptides and proteins from the GI tract

Besides the survival of the protein in the GI tract also protein absorption has to be considered. While it is commonly assumed that dietary proteins are digested to free amino acids in the lumen of the GI tract, there is irrefutable evidence that small amounts of intact peptides and proteins enter the circulation under normal physiological circumstances (GARDNER 1987). Normally, non-allergenic proteins are rarely absorbed as

intact proteins across the intestinal wall at any significant level unless they are specifically designed to do so, such as a special class of milk-borne immunoglobulins (IgA) that protect newborn mammals (GARDNER 1988). It has been shown that < 0.008 % of ovalbumin protein orally administered to humans is detectable in the blood circulation (TSUME et al. 1996).

Intestinal membrane composed of epithelial cells has both transcellular and paracellular routes (POWELL 1981). Protein and peptide absorption is being investigated in the course of oral administration of biopharmaceuticals. From these studies, the paracellular route and the transcellular route are considered as the main permeation route for many drugs and peptides. Enhancers can induce alteration to both the transcellular and paracellular routes, and thus both routes are being analyzed (HAYASHI & TOMITA 1997, 1999).

For example, MCLEAN & ASH (1986, 1987) have reported the time course of appearance of intact (or largely intact) horseradish peroxidase in blood and several peripheral tissues in fish in-vivo. Approximately 0.001 % (rainbow trout) or 0.7 % (carp) of the oral dose of 20 mg was detected in intact form in the tissues examined, which did not include muscle or brain.

Typical markers that have been used for the paracellular route are lactulose and cellobiose (342 Da) (HAMILTON et al. 1987), Cr-EDTA complex (340 Da) (BJARNASON et al. 1983) and polyethylene glycols (average molecular weight 400 to 4000) (CHADWICK et al. 1977).

Disease states in which reversible increases in permeability to these probes have been demonstrated include celiac disease (BJARNASON et al. 1984), Crohn's disease (UKABAM et al. 1983), viral and bacterial gastroenteritis (FORD 1985), parasitic infestations (UKABAM et al. 1983) and alcoholism (PETERS & BJARNASON 1985).

To investigate drug and peptide intake of colon cells, absorption enhancers like sodium caprate (C10), decanoylcarnitine (DC) and organic acids such as tartaric acid (TA) are commonly used. For example sodium caprate (C10), a representative absorption enhancer, significantly increases permeation through both routes in the colon but not in the jejunum (TOMITA et al. 1992).

Given the increasing interest in administering biopharmaceuticals and vaccines via the oral route these studies will continue to provide evidence which could also be relevant for assessing the physiological role of novel proteins.

2.9 Animal studies

2.9.1 Trigger for animal studies

The predominant rationale in GMP guidance documents is that animal toxicity studies on the novel protein are not considered a standard requirement and might not be necessary under certain circumstances. Some guidance documents considered the lack of amino acid homology to known toxins and a rapid degradation in IVD studies as sufficient evidence for safety in toxicity assessment (FAO/WHO 1996). More recent guidance documents suggest toxicity testing on a case-by-case basis depending on the existing database and concerns (KÖNIG et al. 2004, EFSA 2006a).

In practice this could mean that animal studies are required if (i) the specificity and biological function/mode of action of the protein is partly known or unknown or of

concern (for instance a protease inhibitor); (ii) the protein is implicated in mammalian toxicity; (iii) human and animal exposure to the protein is not documented; (iv) modification of the primary structure of naturally occurring forms; (v) when estimated levels of intakes of the new protein indicate adverse effects or (vi) when the protein is not rapidly degraded (KUIPER et al. 2001, KUIPER & KLETER 2003).

2.9.2 Choice of toxicity endpoints

Up to the early year 2000 toxicity endpoints were not mentioned in EU guidance documents (Recommendation 97/618/EC, SCP 1998) and risk assessment dossiers included acute toxicity studies on rodents only¹⁹ (SPÖK et al. 2004), a practice which has repeatedly been criticised (SPÖK et al. 2002, 2003). From 2003 onwards acute toxicity test were disregarded in EU guidance and 28-day repeated dose tests were proposed (KUIPER & KLETER 2003, SSC 2003, EFSA 2006a):

„An acute, single dose test with a 14-day observation period, is inadequate to detect possible toxicity arising from repeated dosing. Furthermore, this test does not provide information on the dose-response relationship and is designed to examine only a few endpoints (mortality, morbidity, clinical observation and gross necropsy) and not the broad range of endpoints required to be investigated in repeated dose studies, such as haematology, clinical chemistry, urine analysis, organ weights and histopathological examination of organs and tissues.“(SSC 2003, p. 19).

While not explicitly disregarding acute toxicity tests KÖNIG et al. (2004) cautions in an influential paper that single dose acute oral toxicity studies may not identify potential adverse effects that might only appear upon repeated dosing.

Toxicity assessment practice, however, did not change. Still, the vast majority of risk assessment dossiers include acute oral toxicity tests only (see Supplement Volume). The few exceptions from this practice are described in Box 3. Sub-acute repeated dose studies were conducted on PAT and the nitrilase protein (14 days) and the Cry34Ab1 and Cry35Ab1 proteins (28 days). Only in the case of the Cry proteins the Panel reviewing the application did ask for a 28-days repeated dose study on top of already provided acute toxicity studies in order to evaluate potential synergistic effects of the mixture of both proteins. Occasionally, repeated-dose toxicity tests are described in the scientific literature (RICHARDS et al. 2003, POULSEN et al. 2007).

This practice is in accordance with guidance given by EFSA if “reliable information can be provided which demonstrates the safety of the newly expressed protein (including its mode of action) and that the protein is not structurally and functionally related to proteins which have the potential to adversely affect human or animal health.” (EFSA 2006a, p. 28). From this it can be interfered that, at least with the proteins from GMPs evaluated so far in the EU there has been no case triggering an 28-day repeated dose study.

90-day subchronic toxicity study in rodents are recommended on a case-by-case basis for isolated substances in novel foods including GM food (EDWARDS 2005, HOWLETT et al. 2003, ROYAL SOCIETY OF CANADA 2001). These testing requirements were, however, designed with non-proteinic substances rather than novel proteins in mind.

The reason why applicants are still providing acute toxicity tests in their dossiers thought these tests were disregarded by EU risk assessors (SSC 2003) can only be

¹⁹ Most of which are single-dose toxicity test. Administering different doses seems to be an exception (e. g. Harrison et al. 1996).

assumed. In the USA, Australia and New Zealand acute oral toxicity tests are still accepted and the EU biotech industry still upholds its recommendation of acute toxicity tests (EUROPABIO 2003). Regulatory documents and dossiers are frequently referring to a conclusion of SJOBLAD et al. (1992) in a paper on *Bt* biopesticides that proteins are known to act via acute mechanisms only. This conclusion was frequently generalised to all proteins (see SPÖK et al. 2002, 2003) and in regulatory science (BRENT et al. 2003, HÉROUET et al. 2005). Another reason might be that repeated toxicity tests require significantly more test protein compared to acute tests (see Section 2.7).

Box 3: Endpoints other than acute oral toxicity in GMP risk assessment dossiers

Acute toxicity tests of the PAT protein via the parenteral route

Rape Ms8 x Rf3 (C/BE/96/01, HÉROUET et al. 2005): The PAT protein was studied by using intravenous injection in mice at dose levels of 1 and 10 mg/kg body weight. The parenteral route of exposure was apparently motivated as it mimics the worst case by considering that 100 % of the entire PAT protein remains intact following ingestion and is absorbed into the body. Furthermore, it allows acute testing with small amounts of proteins (HÉROUET et al. 2005).

14-day repeated dose studies with the PAT protein

Oral toxicity studies with the *bar* encoded PAT protein are not available, but a 14-day repeated dose oral toxicity study conducted in rats fed with the PAT protein encoded by the *pat* gene up to dietary levels of 50000 ppm did not induce toxic effects (5 male and 5 female per group). The test animals received a diet containing 0, 5000 or 50000 ppm PAT protein (i. e. average intake of 0.712 and 7.619 mg/kg/day in males and of 0.703 and 7.965 mg/kg/day in females).

Groups of five male and female Wistar rats (HanIbm:WIST) received diets containing the PAT protein (lyophilized powder) at levels of 0 (group 4), 5 (group 2) and 50 (group 3) g/kg diet. The high level corresponded to a dose of 7.6 and 7.9 mg/kg BW/day for males and females, respectively.

The repeated dose study on the PAT protein was referred to in the dossiers of Rape Ms8 x Rf3 (C/BE/96/01), maize Bt11 (C/F/96/05.10), LLCotton 25 (EFSA-Q-2005-047), maize 59122 (EFSA-Q-2005-045).

14-day subacute toxicity study on nitrilase (AGBIOS database)

According to the applicant in the dossier of canola Oxy-235 (ACS-BNØ11-5) expressing nitrilase an acute oral toxicity study was planned to be performed using doses up to 2000 mg/kg body weight, using a suspension of nitrilase at 200 mg/ml. However, the consistency of the suspension once prepared did not allow the total dose to be administered at one time. Therefore, the suspension was administered over four consecutive days at 500 mg/kg body weight/day. Four consecutive oral doses (500 mg/kg body weight) of nitrilase (Batch No. JHJ0001) were administered to groups of mice (5/sex) at a dose volume of 20 ml/kg. The purified nitrilase was suspended in 0.25 % methylcellulose in distilled water. All animals were checked daily for clinical signs over a period of 15 days, and their body weight recorded weekly (ANZFA 2002).

28-day repeated dose studies with Cry34Ab1 and Cry35Ab1 proteins (EFSA-Q-2005-045)

The EFSA GMO Panel requested the applicant to provide a repeated dose 28-day oral toxicity study in rodents with the Cry34Ab1 and Cry35Ab1 proteins for safety evaluation of synergistic action from kernels from maize 59122 containing Cry34Ab1 and Cry35Ab1 proteins in a specific ratio.

This study included five groups of ten mice each (five females and five males). Three test groups received a mixture of the microbially produced Cry34Ab1 and Cry35Ab1 proteins in the diet at the following doses: 1.97/0.078 (low-dose), 19.7/0.78 (intermediate-dose) or 197/7.8 (high-dose) mg Cry34Ab1/Cry35Ab1 proteins per kg body weight per day, respectively. A control group was fed a standard rodent diet that did not contain the Cry34Ab1 and Cry35Ab1 proteins. In addition, a bovine serum albumin (BSA) protein control group received a diet containing 204.8 mg BSA per kilogram body weight per day to represent an equivalent increase in protein content as in the high-dose Cry34Ab1/Cry35Ab1 group (c).

26-day feeding study on rats of green fluorescent protein (RICHARDS et al. 2003)

Eight weaned rats were fed 1.0 mg GFP each for 26 days, compared to groups fed on a extract of GM rape expressing GFP and other controls. This study does not seem to be conducted according to OECD norms.

90-day subchronic toxicity study of CP4 EPSPS (LEE et al. 2001 cf PETERSON & SHAMA 2004).

45 mg/kg bw and day of the EPSPS protein was administered orally.(b)

a) DFR encodes dihydroflavonol-4-reductase hydroxylase; HF1 encodes flavonoid 3'5'-hydroxylase, both are key enzymes in the anthocyanin biosynthesis pathway from *Pentunia X* hybrid

b) No further details are publicly available.

c) The high-dose level selected for this study was based on a 1000-fold margin of exposure over a worst case scenario for human consumption of the Cry34Ab1 and Cry35Ab1 proteins expressed in maize 59122 grains. The average expression of the Cry34Ab1 and Cry35Ab1 proteins in maize 59122 kernels was ca. 41.9 µg/g tissue dry weight for the Cry34Ab1 protein and 1.66 µg/g tissue dry weight for the Cry35Ab1 protein. Assuming that 100 % of consumed maize (i.e. highest global level of consumption estimated at 4.7 g maize/kg bw/day by the

applicant who refers to WHO GEMS Cluster Diet Data) is derived from maize 59122 and that there is no degradation of the Cry34Ab1 and Cry35Ab1 proteins by food and feed processing, worst case daily human exposure to the Cry34Ab1 and Cry35Ab1 proteins would be 0.197 and 0.0078 mg/kg bw/day, respectively. The doses administered in the 28 day study correspond to 10, 100 and 1000 times this anticipated maximum exposure of humans in the low dose, intermediate dose and high dose group, respectively.

The choice and design of toxicological tests must be proportionate to the nature of the substance under investigation (KÖNIG et al. 2004).

More extensive toxicity testing may be required in cases of (i) a completely novel gene; (ii) novel proteins as anti-nutrients; (iii) novel proteins without a clear threshold (bacterial toxins); (iv) predicted high levels of intake of toxic proteins such as protease inhibitors; and (v) non-rapidly degradable proteins (KUIPER et al. 2001).

Table 12: Toxicity endpoints for protein assessment in guidance documents and the scientific literature.

Endpoints	Trigger/Qualifications	Reference
Acute toxicity	N. sp.	EUROPABIO (2003), FSANZ (2005), ATHERTON 2002, EPA (1998b), SCIENTIFIC INSTITUTE OF PUBLIC HEALTH (2003)
28 day repeated dose (include Tier 1 immunotoxicity screen, Dermal and eye irritation (workers exposed to pollen and crop dust) (a) Genotoxicity (on a case-by-case basis)	Standard requirement?, trigger not specified.	SCIENTIFIC INSTITUTE OF PUBLIC HEALTH (2003)
28 day repeated dose	Toxicity tests necessary in case of insufficient data base or in case of concerns; selected on a case by case basis. Not necessary if safety of protein is demonstrated by other information.	EFSA (2006a), SSC (2003)
Subchronic repeated dose (length of test not specified)	See above	EFSA (2006b) on GMM
Same endpoints as for food additives		ZDUNCZYK 2001

a) Exposure routes other than oral and relevant to toxic action are very rarely ever mentioned in guidance documents and risk assessment dossiers.

b) For Bt proteins only.

Walker argues that subchronic toxicity studies might even be too insensitive to demonstrate the safety of a rapidly digestible protein and short-term tests might therefore be more adequate (FAO/WHO 2000).

As described in the preceding section normally only acute toxicity is tested in rodents. Very rarely, subacute or subchronic oral toxicity is also investigated. To the knowledge of the authors of this report other endpoints have not been investigated.

Box 4: Basic information on toxicity tests required for novel proteins in GM plants and food.

Test	Description	Norms
Acute oral toxicity	Goals: Formerly they were used to identify the LD₅₀ dose (the dose which causes the death of half the test animals) and essentially recorded mortality. At present, they are used to identify a clearly toxic but sublethal dose, to identify major target organs for toxicity, and to provide a rough guide for dose selection in subsequent repeated dose toxicity tests. Relevance in relation to foods and food chemicals: Not particularly useful for hazard identification and risk assessment because human exposure tends to be much lower and to continue over much longer periods. Relevant if acute toxicity is the predominant adverse manifestation	OECD 401, 429, 423
Repeated dose toxicity (28 to 90 days)	Goals: to reveal any targets for toxicity, ranging from organs or tissues to cells, and resulting either from systemic exposure to the test substance or its metabolites, or from direct effects of the test substance on the gastrointestinal tract; identification of dose–response relationships for any targets of toxicity, and establish NOAELs/BMDs. Core parameters include clinical signs, food consumption and body weight gain, ophthalmology and electrocardiography (in dogs), clinical biochemistry and haematology. Besides, gross macroscopic findings, organ weight changes, and microscopic histopathology of organs and tissues are considered. The minimum duration to detect adverse effect is a source of continuous debate. Relevance in relation to foods and food chemicals: Core study, often sufficient to allow for a conclusion in risk assessment	OECD 407, 408
General remarks	Both type of studies do not necessarily address the most common type of acute reaction in humans to food or food chemicals, better described as intolerance rather than toxicity. For example, one of the most common manifestations of intolerance to foods and food components is gastrointestinal disturbance, such as the laxation response to poorly digestible materials. This might be an issue for tolerance studies and post-market surveillance.	

Source: BARLOW et al. (2002)

2.9.3 Relevance of whole-food toxicity studies

The question discussed in this Section is whether whole-food animal studies would also be able to detect adverse effects of the novel protein and if they so would provide complimentary safety assurance to animal studies with the purified protein.

Following a long-standing discussion about the feasibility, relevance and need of whole-food studies in the scientific literature and regulatory science, 90-day subchronic toxicity studies in rodents were eventually included as possibly meaningful and required on a case-by-case basis in recent guidance documents (e.g. CODEX ALIMENTARIUS COMMISSION 2003, SCIENTIFIC INSTITUTE OF PUBLIC HEALTH AND BIOSAFETY COUNCIL 2003, EFSA 2006a). Although not a standard requirement recent risk assessment dossiers frequently include such studies (see Supplement Volume).

The design of more recently conducted studies is based on the OECD norm for 90-day rodent studies (OECD 1995) broadening its scope not only to detect toxic but also adverse nutritional effects (EFSA 2007). The study aims at establishing similarity between a diet containing the GM plant/food and a control containing the conventional crop/food counterpart rather than a dose-response curve.

A number of whole-food feeding studies have been described in the scientific literature and have recently been evaluated by EFSA (2007; see also Supplement Volume). In presently applied animal studies maize is included at about 33 % (w/w) in the diet of

rats, amounting about 33 gram maize per kg body weight per day. Based on an EU Theoretical Maximum Daily Intake (TMDI) about 0.24 g/kg body weight for humans this would allow for a safety margin of about 140. Soybean is typically used at 15 % (w/w), this would amount 7.5 gram soy protein per kg body weight per day. Based on an EU TMDI for soybean of about 0.014 gram per kg body weight per day this would allow for a safety margin of about 500. A similar calculation based on a feeding study with 10 % lyophilised tomatoes equivalent to 200 gram per kg body weight per day resulted in a safety factor of about 700 (EFSA 2007).

Depending on the expression levels for GM maize this would account for 3.3 mg novel protein/kg body weight/day in case of the highest concentration measured in kernel (about 100 µg/g fresh weight). In case of tomatoes and based on expression levels found in tomato for NptII this would account for a dose of about 0.5 µg novel protein/kg bw/day. SCHRODER et al. (2007) calculated the amount of Cry1Ab presenting the GM rice diets of rats in a 90-day feeding study to be about 0.54 mg/kg body weight/day.

Another study design developed in the course of the EU SAFOTEST project applied spiking with the novel protein. A 90-day subchronic feeding study was conducted with GM rice expressing a PHA-E lectin. In the course of an preceding 28-day study diets of 60 % conventional rice mixed with 0.01 %, 0.02 %, and 0.08 % purified PHA-E lectin (the latter corresponding to 55 and 70 mg PHA-E lectin/kg body weight for male and female rats) were orally administered to rats (eight individuals per group). The results of the study were used to identify the toxic effects of the pure PHA-E lectin and to find the LOAEL for the most critical one, which then was used to select the spiking level for the pure lectin in the 90-day study. In the 90-day study rats were fed on a diet prepared from GM rice expressing the lectin, the diet for one group was spiked with 0.1 % PHA-E (corresponding to a dose of 70 mg PHA-E lectin/kg body weight. Together with the PHA-E lectin contained in the GM rice this results in a total amount of lectin ingested of about 100 mg PHA-E/kg body weight (POULSEN et al. 2007).

The research team concluded that this test design “is sensitive and specific enough to verify the presence/absence of the biological, nutritional and toxicological effects of the novel gene insert at the level of intake defined in the test and within the remit of the test” (KNUDSEN & POULSEN 2007).

Compared to single-substance toxicity testing whole-plant/food studies seem to provide several advantages. A repeated-dose design of 90-days is definitely more representative of human exposure to a substance included in the diet compared to acute testing. With the exception of spiking, the novel protein is the original plant protein and there are no protein purification steps needed which could alter protein properties. The novel protein is presented to the human GI tract in a form that much better resembles the diet of humans compared to oral gavage of a highly concentrated protein paste. In case some kind of processing of the animal diet would be required to avoid interference from plant endogenous anti-nutrients or toxicants (e.g. soybean, tomatoes) this would also be the case for human food. Any effects either of the food matrix or of food processing mitigating or increasing toxicity level can also occur with food processed for the human diet. Eventually, the MoS which can be calculated from the NOELs observed in these test designs seem to be well beyond 100 and might therefore be considered acceptable and also apply for the novel protein included in the diet. Thus, it can be even be asked if whole food/plant studies would be more sensible compared to acute toxicity studies of the isolated protein.

There are, however, several limitations to draw conclusions on novel proteins from these studies.

Based on a database of MUNRO et al. (1996) EFSA has estimated that LOELs based on oral studies in rats of 121 chemicals, pharmaceuticals, and food substances range between 0.2 and 5000 mg/kg body weight/day, with a median of 100 mg/kg body weight/day (EFSA 2007). EFSA used this range for estimating the minimum concentration of a plant substance that allows for a detection of toxic effects. Applying these considerations to plant-expressed novel proteins the diets prepared from GM tomato and GM rice described above might include sufficient protein to detect adverse effects. Following the considerations in the EFSA document (ibid) this might however only be possible for proteins with a significant toxic or anti-nutritional potential. For proteins of low or no toxicity it would not be possible to establish a LOEL and therefore also the feeding studies would provide useful information on the novel protein. Spiking of diets in one group might be possible to increase protein concentration in case of low-level expression in the plant but might encounter the problem of relevance as the protein material from spiking has so far been derived from microbial sources (see also discussion in Section 2.8).

Whether the calculated MoSs would also apply for the novel protein depends on several preconditions. In order to properly calculate the dietary intake of the novel protein one would have to measure the concentration of the novel protein in the diet and establish that the protein remains functionally intact. Measuring the concentration of the novel protein might, however, be difficult: routinely used methods such as ELISA do not discriminate between functionally intact and (partly) denatured proteins. Activity based methods would require isolation of the protein which itself can influence protein structure and activity. Thus only in case of spiked diets there is sufficient certainty of the presence of a certain minimum level of intact protein. As spiked proteins are derived from microbes, here, again the question of equivalence comes up.

In case of preparing a diet from raw plant material it can perhaps be assumed that the novel protein is functionally active. In case of potentially protein denaturing steps (e.g. heating and shift in pH) a partial or complete degradation might be possible. However, there are large variations in food processing and one particular model processing might not be representative for all variations of human food prepared from one particular crop (with respect to the fate of single substance in the food).

Another limitation would be the interpretation of results. In case of adverse effects – unless this would result in a full stop of development of a particular GM crop – additional studies would be needed to establish whether the effects are caused by the novel proteins or any indirect effects. If no adverse effects are detected, diet preparation and food matrix etc. could mitigate any toxic or otherwise adverse health effects. Such adverse effects could well show up if the protein is expressed in other GM event/plant species context or in case of different ways of preparing the diet.

Therefore, it seems that whole food toxicity studies could provide additional safety assurance in the course of protein toxicity assessment but needs careful interpretation depending on the particular case and the design of the study. It is, however, unlikely that they could replace toxicity studies of the novel protein.

2.10 Exposure assessment

Exposure assessment is an essential part of risk assessment and is required in guidance documents on GM plants and food (e. g. CODEX ALIMENTARIUS COMMISSION 2003, SCIENTIFIC INSTITUTE OF PUBLIC HEALTH AND BIOSAFETY COUNCIL 2003, EFSA 2006a).

SPÖK et al. (2003, 2004) described what kind of exposure relevant information is included in EU risk assessment dossiers. As a general insight for this review, exposure

relevant information provided differ between dossiers, even in cases of the same plant species where a similar kind of application in food would be expected. In general exposure is only considering the oral route. Worker's exposure to dust etc. was not considered in risk assessment dossiers up to 2001. Some of the more recent dossiers briefly mention occupational exposure in the context of allergenicity assessment only (results not shown). Table 13 provides an overview of exposure relevant information included in the dossiers.

Exposure assessment in the course of GM plant/food risk assessment is not only relevant for risk characterisation of the novel protein. It is also relevant with respect to risk characterisation of the whole plant (e.g. based on the results for whole food toxicity studies) and could be of relevance for nutritional considerations.

Table 13: Exposure relevant information in GM plant and food risk assessment dossiers.

Information in risk assessment dossiers	Frequency	Notes
Concentration of the novel protein(s) in the plant tissue used in food processing (EFSA 2006a: variance and means)	Routinely provided	
Description and of food processing (EFSA 2006a: influence of processing, storage and treatment of food)	Routinely provided	Huge differences in the degree of details and comprehensiveness
Information on the stability of the enzyme to high temperatures	Occasionally	
Estimates/measures of concentration of the novel protein(s) in final food	Rarely	
Information from IVD studies	Routinely provided	
Considerations of population subgroups which might be especially affected/concerned (high risk/high intake)	Occasionally	Very briefly
Consideration of any changes in consumption pattern following market introduction	N. r.	Very briefly
Estimates of dietary intake	Very rarely	

N. r.: *So far, not particularly relevant to first generation of GM crops.*

More recent risk assessment dossiers seem to follow a similar pattern (see also overview of exposure relevant information in Supplement Volume). Information focuses on the concentration of proteins, description of processing, IVD tests, and occasionally thermostability testing.

Information on the concentration of proteins in food products is rarely provided and if so it is about the absence of proteins in certain kind of foodstuff.

Dietary intake is occasionally considered. For instance, the dossier on maize 59177, which is intended to be used for food and feed and which expresses three novel proteins: Cry34Ab1, Cry35Ab1 and the PAT.

- In order to estimate the dietary intake it is assumed that 100 % of consumed maize is derived from maize 59122 maize and it considers that the Cry34Ab1 and the Cry35Ab1 proteins occur in all maize, maize products and unspecified cereal products.
- Maize: Based on a consumption of 15.4 g maize/person/day (according to WHO Global Environment Monitoring System (GEMS) Cluster Diet). This relates to a theoretical maximum daily intake (TMDI) of 0.25 g maize/kg bw/day.²⁰

²⁰ Assuming a body weight of 60 kg.

- Cry proteins: Based on a TMDI of 0.25 g maize /kg bw/day the TMDI for the Cry34Ab1 and Cry35Ab1 proteins calculated for European consumers, equals to 0.012 mg/kg bw/day and 0.00042 mg/kg bw/day respectively. This TMDI is calculated based on mean Cry34Ab1 and Cry35Ab1 protein expression levels in 59122 maize grain of 0.04617 mg/g of grain dry weight and 0.00168 mg/g of grain dry weight respectively.
- The mean expression level of the PAT protein in 59122 maize grain is calculated as 0.000051 mg PAT protein/g grain dry weight. Taking into account that maize for human food use in Europe represents blended products and that most maize is consumed as commodities, such as high fructose maize syrup, maize oil and starch, that have negligible amounts of protein, the daily intake of PAT protein will be negligible.

Other recent examples, however, do not provide any description of exposure and estimates of dietary intake (e.g. maize stacks NK603xMON810, MON863xMON810, MON863xMON810xNK603).

Another example is the dossier on maize 3272. This GM maize expresses both a thermostable amylase and the PMI protein (as selective marker) and is intended to be used in the dry-grid ethanol production process. By-products of the process will be used as feed. Application as food is not intended but might happen following commingling with food maize. Though it is taken into account by the applicant that event 3272 may enter the food chain, no statements about the amount of possible daily intake – neither by man nor by animals can be found in the dossier.

2.11 Risk characterisation

Risk characterisation represents a final step in risk assessment combining results from hazard identification and characterisation and from exposure assessment. According to LEEUWEN & HERMENS (1995) risk characterisation includes, in principal, comparing the total intake to a toxicological parameter preferably derived from long-term or at least sub-chronic studies. The most frequently used parameter for non-genotoxic substances is the NOAEL (no observable adverse effect level). The studies selected are usually tests with experimental animals for which a NOAEL_{mammal} is derived. If reliable human data can be used to derive a NOAEL, this value is preferred. Where a NOAEL cannot be derived, a LOAEL, if available can be used. Exposure can, in principal, be judged to be acute, subchronic or chronic related to the exposure schedules in experimental studies.

The direct comparison of toxicity data with exposure gives an indication of the degree of risk in terms of a margin of safety (MoS), which is a quotient of NOAEL and total exposure. Depending on the biological species for which the NOAEL has been derived (man or animal), the exposure period and frequency, the incidence, type and severity of the effect it can be judged whether the resulting margin is sufficient or not. This margins should also take into account uncertainties due to intra-species variations, i.e. variations in sensitivity among individuals of one species, as well as interspecies variations (between man and the test animal). Additionally uncertainties resulting from differences in the exposure scenario, e. g. short-term versus long-term exposure and continuous versus intermitted exposure. The margin should take into account the uncertainties in the estimated daily intake or uptake.

Risk assessment in the context of GM plants and food is in principle following this approach with risk characterisation understood as “the quantitative or semi-quantitative estimate including attendant uncertainties, of the probability of occurrence and severity of adverse effects in a given population under defined conditions based on hazard

identification, hazard characterisation and exposure assessment” (EFSA 2006a referring to SSC 2000).

In risk assessment dossiers up to 2001 risk characterisation based on hazard assessment of and exposure assessment with the novel protein(s) was hardly ever conducted in such a systematic way (SPÖK et al. 2002, 2003, 2004).

The main reasons are that

- Normally only acute toxicity studies are being conducted from which no NOAEL/NOEL were derived.
- No proper estimates on dietary intakes were included.

More recent dossiers are partly similar to those, essentially not including a description of exposure and estimates of dietary intake (e.g. maize NK603xMON810, MON863xMON810, MON863xMON810xNK603). No NOEL was derived in these cases. Risk characterisation was limited to point to several years of consumption of GM plants containing these proteins and to refer to the absence of adverse effects on humans health.

Other dossiers calculate a NOEL based on single-dose acute toxicity study in rodents (e.g. maize MON 863 and MON863xMON810, maize MON863xNK603). Normally, no indication of toxicity is being reported despite the high doses of up to 5000 mg/kg bw. This dose is then considered to be the minimum NOAEL and used to calculate a safety margin.

As mentioned above and according to standard toxicology a NOAEL should not be derived from acute tests, in particular if the exposure would be long-term rather than acute.

Some authors however justified these practice under certain conditions:

“given the established database indicating that microbial toxins that are active via the oral route are also acute toxins (see text), we conclude that in this very limited case, for the purposes of enzyme safety evaluation, a NOAEL can be determined from short-term gavage/feeding tests” (PARIZA & JOHNSON 2001, p. 181)

BARLOW et al. (2002) also contended that in exceptional case where substances are known to act only via acute mechanisms this approach might be acceptable.

There are very few exemption to this found in practice, e. g. maize 59122. From 28-day repeated dose tests in mice it was concluded that there were no treatment-related effects in clinical signs, to the Cry34Ab1/Cry35Ab1 proteins at any of the dose levels tested. The highest dose tested of 197 and 7.8 mg/kg bw/day for the Cry34Ab1 and Cry35Ab1 proteins respectively were therefore considered as NOELs. Based on these numbers and a TMDI for maize, a MoS greater than 1000 was calculated. The repeated dose study was, however, only provided on request of the EFSA GMO Panel (in order to evaluate any synergistic effects of the two related proteins).

Another strategy found in risk assessment dossiers is to argue that either exposure would be virtually zero or that the hazard is considered to be zero. For instance in case of maize T25 it was argued that no active proteins will remain after processing of the maize. The applicants argued that there is no need for toxicity testing at all. Conversely, if the toxicological risks are shown to be negligible (e. g. in case of maize NK603), the risks are characterised without considering exposure (SPÖK et al. 2003).

More recent risk assessment dossiers do however frequently include a 90-day subchronic feeding study on the whole plant. As described in Section 2.9.3 NOAELs are derived from such studies allowing for MoSs from about 100 to 700. However, as

contended in a recent EFSA draft document (EFSA 2007) these kinds of feeding trials is not considered sufficient to detect adverse effects of proteins which are of low toxicity.

2.12 Summary

Toxicity assessment of novel proteins in GM plants and food as outlined in most guidance documents follows a case-by-case approach. In practice, hazard identification and characterisation essentially includes information on the history of human exposure/consumption, information on the source organism, the mode of action and specificity, potential homology to known toxins, in-vitro digestibility studies and acute toxicity studies in rodents. Exposure assessment includes information on expression levels in the plant and description of food processing including studies on temperature stability. A calculation of dietary intake of the novel protein based on food consumption databases and expression levels is not always provided.

According to recent guidance documents in case there exists a **history of safe consumption** of the introduced protein toxicity tests might be waived. A history of safe consumption frequently refers to anecdotal evidence and often to generic categories such as EPSPS proteins or Cry proteins “in general”, or technical grade **Bt** products. The absence of negative health impacts is usually not documented by epidemiological studies, thus perhaps missing out long-term and/or less severe effects. Moreover, even the anecdotal evidence is frequently limited to a few sentences. Despite its key role the concept of history of safe use has not been defined and is lacking scientific precision.

In the **description of the source organism** a precise taxonomic identification is prerequisite to track reports of any toxic or pathogenic properties which could have a bearing on the function of the isolated gene. Experience with source organisms are, so far, limited to microbial sources many of which are claimed to have some kind of history of human exposure.

The **mode of action**, understood as the physiological role of the protein and its underlying molecular mechanisms, and the specificity can be investigated for its relevance to human physiology. The mode of action is perhaps most extensively investigated with Cry proteins. Still, 12 years after market commercialisation questions on the biochemical activity of **Bt** toxins in humans are being raised.

Information on the type of **food processing** can be relevant for the likelihood of novel proteins being present in the final food product. Food processing often includes heat treatment and shift in pH which both could inactivate food proteins. This is also the main reason for providing information on temperature stability though it is not always clear how these studies relates to the most relevant routes of food processing.

Amino acid sequence comparisons can establish similarity/homology to known toxins, the absence of which is taken as a confirmation of safety. In contrast to homology searches for allergenic epitopes there is no detailed guidance available for conducting such comparisons. More recent practice the homology search approach employed in allergenicity assessment (35 % similarity within 80 consecutive amino acids) is also used in toxicity assessment. Homology comparisons reviewed in the course of this study differ in algorithm, parameters and databases used. Fast methods seem to be preferred to more sensitive methods. Some sequence comparisons have been conducted more than a decade ago and are still being referred to in recent risk assessment dossiers despite the fact that the number of sequence entries of proteins suspected or known to have toxic properties have dramatically increased. According to company information homology searches are updated every year or every three years

depending on the company but not submitted and also not always included in the submission for renewal.

The **test protein** used in animal studies and occasionally also in in-vitro digestibility (IVD) studies is normally purified from micro-organism. This practice has been justified with the very low levels of protein expression in plant tissue. As plant and microbial proteins can differ with respect to amino acid sequence, folding, post-translational processing, e.g. glycosylation structural and functional equivalence of test protein and plant derived protein has to be established. This adds additional uncertainties to the interpretation of animal toxicity tests. An estimate of the amount of test protein needed revealed that in case of expression levels beyond 100 µg/g fresh weight up to 28-day subacute studies might possibly be conducted with plant-derived protein.

In-vitro digestibility (IVD) tests are considered to provide evidence on the fate of the protein in the human gastrointestinal (GI) tract following ingestion. A comparison of the experiments described in risk assessment dossiers and in the scientific literature revealed considerable variations in the protocols with the amount of pepsin used in SGF per unit test protein being most striking. In the absence of more detailed guidance IVD studies are likely to lead to arbitrary results. Furthermore, the relevance of IVD for the in-vivo situation appears to be questionable. Different to single substance digestion in-vitro, in-vivo proteins can be protected in a food matrix, GI conditions can differ depending on the type of food, on age, stomach content, existing pathologies and medication. Contradicting evidence is so far available from animal studies confirming both rapid digestion and detectability of the novel protein in the GI and faeces of mammals. This is reflected in different and contradicting views on IVD studies in guidance documents and the scientific literature. More complex models have therefore been proposed by some authors. Neither the SGF and SIF models nor any of the more complex in-vitro models have, however, been validated so far.

Animal toxicity studies are recommended on a case-by-case basis and might not be required if other evidence suggests that the protein is safe. Toxicity studies might be required and the selection of endpoints might be influenced if (i) the specificity and biological function/mode of action of the protein is partly known or unknown or of concern (for instance a protease inhibitor); (ii) the protein is implicated in mammalian toxicity; (iii) human and animal exposure to the protein is not documented; (iv) modification of the primary structure of naturally occurring forms; (v) when estimated levels of intakes of the new protein indicate adverse effects or (vi) when the protein is not rapidly degradable. Toxicity endpoints proposed differ across guidance documents, including acute toxicity or 28-day subacute oral toxicity test with acute toxicity tests being explicitly disregarded by some. In practice however, with rare exceptions only single-dose acute toxicity tests were conducted.

90-day whole plant/food subchronic studies in rodents can provide additional safety reassurance in the course of protein toxicity assessment depending on the particular case (design of the study, nature of the protein, GM plant species etc.).

Exposure assessment and risk characterisation should – according to recent guidance documents - follow the same rationale as in other regulatory contexts. Estimates of daily intake based on comprehensive database of food consumption is only included in recent risk assessment dossiers. In case a more formal calculation of the margin of safety (MoS) is included, a NOAEL is sometimes derived from acute toxicity tests. This does not correspond to normal toxicological practice, which foresees a NOAEL to be calculated based on a minimum of 90-day subchronic studies. Alternatively, it is being argued in risk assessment dossiers, that either exposure (e.g. no protein detectable in the food stuff) or toxic properties (e.g. no toxic properties detected in maximum-dose acute toxicity tests) are negligible and therefore there is no risk anyway.

3 Protein Toxicity Assessment in Other Regulatory Contexts

Besides GM plants and food proteins have routinely been assessed for their toxicity only in the context of market authorisations of food and feed (food and feed enzymes), biopesticides, chemicals and pharmaceuticals. Occasionally, proteins and polypeptides have also been assessed as food additives (e.g. lysozyme, thaumatin), novel foods (e.g. ice structuring protein) and cosmetics (e.g. certain enzymes).

The vast majority of these proteins are enzymes which are used in food, feed, cosmetics, as pharmaceuticals, diagnostics as well as chemicals for research and production in a broad range of industrial contexts. Protein toxins are used as biopesticides (e.g. *Bt*-toxin), in human therapy (e.g. ricin) and in cosmetics (e.g. botulinus toxin). A range of hormones, interferons and cytokinin are used as pharmaceuticals and virus protein are being produced as vaccines.

Only few numbers of enzymes regulated under EU chemical legislation have been subjected to a more extensive toxicological testing (FEDERAL ENVIRONMENT AGENCY & IFZ 2002). From our own reviews of feed enzyme testing in the EU it become evident that only a few oral toxicity studies have been conducted. Most feed enzyme dossiers include tolerance studies in the target animal rather than toxicity studies (SPÖK, unpublished).

Biopharmaceuticals would represent an interesting area as a broad range of toxicological data is asked for in pre-clinical studies. Since 1982 more than 142 biopharmaceutical products have been launched and about 300 more are presently in clinical trials (and therefore must have been tested) (ZİKA et al. 2007). Given the high attrition rates in the development of biopharmaceuticals the number of toxicity assessment of pharmacologically active proteins might be much higher. However, with few exceptions these assessments are usually not publicly available. Furthermore, most of the biopharmaceuticals are applied parenterally and the design of toxicity test is chosen accordingly.

For reasons of relevance and feasibility this Chapter therefore focuses on the contexts food enzymes and biopesticides.

This Chapter describes protein toxicity assessment as in other regulatory context than GM plants and food, food enzymes (see Sections 3.1) and biopesticides (see Section 3.2). The description focuses on guidance requirements and regulatory practice. Guidance requirements for food enzymes are also comparatively analysed in the Supplement Volume (see Tables 10 and 11).

3.1 Food enzymes

In terms of EU food regulation enzymes have so far been distinguished into food additives and processing aids²¹. The main distinction between food additives²² and

²¹ Theoretically enzymes might also be considered as food ingredients. In this case enzymes from GMO would be regulated by the Regulation (EC) No 258/97 of 27 January 1997 concerning novel foods and novel food ingredients. However, in practice this is not done.

²² Food additives „means any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods.” (Directive 89/107/EEC, Article 1.2).

processing aids²³ is that additives do have a technological function in the final food whereas processing aids do not. Processing aids are applied during the manufacturing process of foods. Most food enzymes are considered as processing aids only two enzymes, lysozyme and invertase were authorised as additives following an safety evaluation by the SCF. So far, only the regulation of food additives has been harmonised in the EU, an EU regulation on food enzymes in general including processing aids is pending.

Consequently, there is so far little experience in assessing food enzymes at the level of the European Commission's scientific committees.

Most assessment has been voluntarily conducted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). JECFA is carrying out evaluations of food additives which normally lead to the calculation of an acceptable daily intake (ADI) and to the development of specifications for identity and purity. JECFA evaluations are acknowledged by many countries worldwide.

Non-enzymatic proteins used as food additives

Besides enzymes only very few proteins are used as food additives.²⁴

One example is Thaumatin. Thaumatin is extracted from the West African Katemfe fruit *Thaumatococcus daniellii* and consists of a mixture of proteins with a molecular weight of about 22 kDa. Thaumatin (E957) is authorised as sweetener under Directive 94/35/EC on sweeteners for use in food²⁵ and as flavour enhancer in chewing-gum, beverages and desserts under the Miscellaneous Additives Directive 95/2/EC. Thaumatin is also approved in all applications in the EU as a "flavour preparation" under Directive 88/388/EC.

The SCF conducted evaluations of thaumatin in 1984 and 1989.

Thaumatin constitutes a rather unusual food additive in so far it is a protein. It cannot be anticipated in which way proteins that are produced and applied in food for other than enzymatic function would have been evaluated for their safety. There is definitively no particular Guidance available that would cover such case. Thus, this kind of substances will be excluded from this chapter.

Guidance on safety evaluation are provided by the SCF, on food additives in general (SCF 2001) and on enzymes in particular (SCF 1992).

So far, 5 enzymes were evaluated by the SCF and one by its successor within EFSA, the AFC panel (see Table 11, Supplement Volume). Additives authorised under Directive 95/2/EC are listed in the annexes to this Directive which are regularly updated. Only two enzymes lysozyme and invertase are authorised as food additives in the EU (see Table 8, Supplement Volume).

The opinions on urease were issued in the context of specific food legislation, Commission Regulation (EC) No 1622/2000 which permits and sets out criteria for the use of enzymes for wine making. Invertase, and Papain as well as the activated

²³ Processing aids „means any substance not consumed as a food ingredient by itself, intentionally used in the processing of raw materials, foods or their ingredients, to fulfil a certain technological purpose during treatment or processing and which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final product, provided that these residues do not present any health risk and do not have any technological effect on the finished product.“ (Directive 89/107/EEC, Article 1.2).

²⁴ Polypeptides (e.g Nisin, 34 amino acids) and oligopeptides are not considered in this study.

²⁵ European Parliament and Council Directive 94/35/EC on sweeteners for use in foodstuffs, amended by Directives 96/83/EC and 2003/115/EC.

lactoperoxidase system were not granted a favourable opinion. Toxicity data were evaluated in the context of chymosin and urease only.

3.1.1 SCF-Guidelines

Requirements for toxicological data are dealt with in the SCF Guidance on submissions for food additive evaluations (SCF 2001). Section 9 is dealing with additives from microbial processes including GMM. Section 10 is on additives produced from GMO. In the latter case the Guidance requires additional information according to Recommendation 97/618 or subsequent Guidelines.²⁶

This Guidance was however not prepared to evaluate proteins, rather it is focussing on chemically defined single substances (e.g. sorbic acid), chemically defined simple mixtures (e.g. lecithin), complex mixtures (e.g. xanthophylls), and polymers (etc. starches, xanthan) (ibid: 6–7).

Enzymes have so far been evaluated by the SCF Guidelines for the presentation of data on food enzymes (SCF 1992). These guidelines include conditions of use, requirements for information, documentation and testing and do occasionally refer to concrete methods of testing. In doing so the SCF did not discern between processing aids and additives.

Consequently, these guidelines are applicable and obviously have been applied to both types of enzymes. Nevertheless, as evaluation of processing aids is not mandatory the number of evaluations conducted so far is quite limited (see Table 7 in the Supplement Volume).

The guidelines focus on enzyme safety evaluation and consider themselves as minimum requirements for information to be supplied. These requirements refer to potential hazards and to the exposition of the enzyme preparation to the final consumer. With respect to hazards, the guidelines are mainly focussing on toxicological requirements of enzyme preparation, on the safety of the source organism and on unintended reaction products in the food caused by enzymatic reactions in the final foodstuff. Exposition is mainly dealing with the quantity of enzyme consumed. Immunogenic or irritative effects are just briefly mentioned by stating that these effects are considered primarily as occupational problems.

A detailed list of information requirements is given in Table 10 (Supplement Volume). Whereas the SCF considers enzymes from (edible) plants or animal species as posing no health problems, the toxicological evaluation of enzymes from microbial sources is deemed to be far more important. These toxicological tests should investigate known toxins as well as unknown toxic compounds that might be present in the enzyme preparation.

Exemptions from full testing requirements may be justifiable if the production strain of an already tested and approved enzyme preparation is substituted by a mutant strain²⁷ or in case of highly pure and specific enzyme preparations (which will become possible due to the use of non-toxin-producing GMM as hosts).

An enzyme preparation may even be accepted without specific toxicological testing if the production organism has a long history of safety in food use, and belongs to a

²⁶ From Footnote 4 it can be concluded that presently the EFSA Guidance on genetically modified food and feed (EFSA 2004) and/or the proposed Guidance on genetically modified microorganisms and their derived products (EFSA 2005) would have to be considered instead.

²⁷ Whether the term „mutant strain“ also includes GMM is not specified.

species where no toxins are produced, and if the particular strain is of well documented origin.

The SCF (now the AFC) has to ascertain the safety in use of the particular enzyme preparation. Thereby either acceptable conditions for use will be described or an ADI based on the NOAEL in the sub-chronic rodent study with the application of a suitable safety factor will be allocated.

The evaluations are regarded to be confined to a particular enzyme preparation described in the submission and cannot „automatically be considered to cover other preparations of the same enzyme prepared from other sources or by other processes” (p. 21).

Accordingly, whether an enzyme preparation is regarded as being new/different to an already approved enzyme preparation might be considered on a case-by-case basis: e. g. if changes in the manufacturing and purification process would result in an enzyme preparation that does/does not substantially differ from the original one. These changes may also include the replacement of the production strain by a mutant.

3.1.2 EFSA Guidance on GMM

Once the new EU regulation on food enzymes will be adopted EFSA will also compile a guidance for pre-market risk assessment requirements of food enzymes. A recently published “Guidance Document for the Risk Assessment of GMMs and their Derived Products Intended for Food and Feed Use” (EFSA 2006b) covers a similar area and might therefore be instructive for food enzymes. This document primarily provides guidance for the risk assessment of GMMs and/or derived food and feed within the framework of Regulation 1829/2003. Food additives in the meaning of Directive 89/107/EEC produced from/with GMM are also covered. Table 11 (in the Supplement Volume) compares the requirements for toxicity data of the SCF Guidance document (1992) to the recent EFSA Draft Guidance.

The rationale of the EFSA Guidance on GMM is very similar to the EFSA Guidance on GM crops and food, allowing for a case by case approach depending on (i) the source, function and history of human/animal consumption of the protein; and (ii) on comparisons to conventional counterpart, even in case of single substances. The risk assessment requirements include homology comparisons and IVDs. Animal toxicity studies might not be required if both the GMM and the protein have a history of safe consumption and/or, if there is other information which suggests that there is no concern (no homology to proteins which can exhibit adverse health effects, sufficient information on the mode of action etc.). If animal studies are considered necessary subchronic toxicity studies should be conducted the duration of which is not specified. Further toxicity studies would then depend on the outcome of the subchronic study.

3.1.3 JECFA Guidelines

For food additives, contaminants and naturally occurring toxicants, JECFA (i) elaborates principles for evaluating their safety; (ii) conducts toxicological evaluations and establishes acceptable daily intakes (ADI) or tolerable intakes; (iii) prepares specifications of purity for food additives; and (iv) assesses intake. The Committee accomplish their task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs.

JECFA guidelines on food enzymes have been published and further revised since 1971. In addition to these enzyme specific guidelines the „Principles for the safety assessment

of food additives and contaminants in food" (WHO 1987) specified especially toxicological testing requirements.

A (temporary) approval of enzymes from the Committee results either in the allocation of an ADI or – more often – in general statements that, e. g. in the opinion of the Committee, the enzyme preparation does not represent a hazard to health (for that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary).

3.1.4 Perspectives from industry

As the safety evaluation of enzymes used as processing aids is not regulated in most EU Member States industry has also set up their own guidelines acknowledging the guidelines for safety evaluation JECFA (AMFEP 1992), SCF and COT (AMFEP 1996). Information and documentation which must be available for a detailed review and assessment of the safety of use according to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) can be seen in (see Table 10 in the Supplement Volume).

AMFEP also established criteria to develop a list of microorganisms that can be recognized as safe for food production (AMFEP 1992). The safety of production organisms is considered key for all kind of possible health risks and it also reduces the likelihood of inducing the production of known or unknown toxins.

3.1.5 Regulation of food enzymes in the US

Enzymes used in food are regulated by the United States Food and Drug Administration (FDA) under the Food, Drug and Cosmetic (FDC) Act. Since 1958 the FDA has perceived enzymes as food additives and therefore subjected to the restriction pertaining food additives. In general food enzymes may either be classified as (i) substances that are „generally recognised as safe" (GRAS), (ii) substances that are not GRAS which are defined as food additives, or (iii) substances approved for use in food prior to September 6, 1958 by the FDA or by the Department of Agriculture. The FDC Act requires approval of food additives prior to marketing. GRAS substances, in contrast, are not subjected to approval or notification to the FDA prior to marketing.

GRAS status may be based either on a history of safe use in food prior to 1958 or on scientific procedures which require the „same quantity and quality of evidence as would be required to obtain a food additive regulation". GRAS status may be either affirmed by the FDA or determined independently by qualified experts.

Detailed recommendations for enzymes are given in a guidance document (FDA 1993).

These recommendations intend to aid petitioners in assembling the chemical and technological data currently considered appropriate for a food additive or GRAS affirmation petition for an enzyme preparation. They cover data requirements in the following areas: identity, manufacturing process, purity, use, analytical methodologies, technical effects, and probable human exposure (shown in Table 10, Supplement Volume). The recommendations do not address other data needs, such as those pertaining to microbiological, toxicological and environmental considerations.²⁸

²⁸ The extent of toxicological testing of food additives is depending on the on the assignment of a „concern level", on structural features and on an estimation of exposure. Minimum testing requirements are recommended for each concern level as well as each structural and exposure group (FDA 1997). However, these requirements are not investigated in the course of the project.

In order to evaluate the safety of an enzyme preparation, the petitioner and FDA are comparing the enzyme to be assessed with other enzymes which have already been approved or with those that have been „safely consumed as part of the diet throughout human history“. According to FDA „enzymes that have the same function and that are identified by the same name and EC number often differ slightly in structure and properties when they are obtained from different sources. For example, the structure of an enzyme isolated from one tissue (such as liver) of one animal species, may differ slightly from that of the same enzyme isolated from a different tissue from the same species, or from the liver of another animal species. In part because of this variability, the diet routinely contains many thousands of different protein molecules“ (FDA 1995).

This approach is based on the concept of Substantial Equivalence (in earlier documents also referred as substantial similarity) which is also used in the safety evaluation of novel food. According to the definition if „a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety. No additional safety concerns would be expected.“

Consequently, the food or food component can be concluded as safe as conventional food or food component (OECD 1993, FAO/WHO 1996). This concept was further recommended by international expert groups to be applied in the assessment of „substances intentionally added to food.“ For example, a carbohydrase preparation and a protease preparation from *Bacillus subtilis* and *Bacillus amyloliquefaciens* were assessed to be substantially equivalent to carbohydrase and protease enzymes from other microorganisms that have been evaluated and found to be safe by FDA before (e.g. mixed carbohydrase protease preparation from *Bacillus licheniformis*, carbohydrases from *Rhizopus niveus*, *Rhizopus oryzae*, *Aspergillus niger*) (FDA 1999).

3.1.6 Comparison of toxicity assessment requirements for food enzymes

In Table 10 (in the Supplement Volume) the guidelines from SCF, JECFA, AMFEP, and FDA are compared with respect to toxicity assessment and characterisation of the enzyme protein. Similarities include:

- basic technical data on the enzyme itself, as the active compound of the preparation,
- information on the source material; special attention is given to microbial sources,
- additional information in the case of GMM (host organism, donor organism, vector, introduced DNA).

In general guidelines and provisions do not precisely describe the requirements. Thus, much is left to interpretation and can only be taken from published safety evaluations. Most detailed technical information on the enzyme is required by the FDA (enzymatic function, mode of action, substrate specificity, molecular weight, isoelectric point, kinetic properties, specific activity, temperature, pH, inorganic ions). In general, more detailed information is requested in case of GMM.

FDA requires the most detailed information on enzymes from GMM, including detailed technical data on the enzyme itself, on structural modifications, on the GMM (genetic stability, growth properties), vector, introduced DNA, donor organism as well as more detailed information on the manufacturing and purification process. Also the JECFA guidelines require more detailed technical data on enzymes from GMM whereas the SCF asks in greater detail on the vector. FDA also specifies a list of parameters which should be used in comparative analysis to justify substantial equivalence (enzymatic activity, kinetic parameters, amino acid composition, amino sugar composition, amino acid

sequence, molecular weight, isoelectric point, gel-migration, chromatographic properties).

SCF describes basic toxicological requirements, that are toxicological tests to be performed and possible exemptions from testing within the enzyme guidelines. The JECFA laid down general principles for toxicological testing in a separate publication. The particular requirements for toxicological testing are primarily depending on the nature of the microbial source, e.g. if it is a microbe that also naturally occurs in food, if it is a GMM etc. Unlike the contested proposals for toxicity testing in the scientific literature (PARIZA & JOHNSON 2001), toxicity endpoints suggested in guidance documents are fairly similar. SCF and JECFA are asking for a 90-day subchronic toxicity test along with mutagenicity tests on bacteria and an in-vitro test for chromosomal aberration (see Table 14). AMFEP does not specify particular toxicity endpoints for food enzymes. Only SCF includes requirements on data for the manufacturing process, the usage and stability in food.

Detailed descriptions of methods or references for methods to be used in testing and data production are given by both SCF and JECFA.

The AMFEP guidance does in general not include detailed description of requirements. However AMFEP does acknowledge the guidelines from JECFA, SCF and COT. Safety approvals are often confined to particular enzyme preparations (SCF). Changes in the production process or application of genetic engineering lead to a re-evaluation on a case-by-case basis. Waivers from toxicological testing are also dealt with on a case-by-case basis.

FDA is taking into account long-term experience with certain enzymes. Such enzymes may be assigned the status of GRAS.

Table 14: Toxicity endpoints proposed for food enzymes derived from microorganisms.

Toxicological endpoints/References		Rasmussen & Skovgaard (1998)	Pariza & Johnson (2001)	SCF (1992)	EFSA (2006b)	JECFA
Acute toxicity (oral)		n.r.	Ya	n.r.	n.r.	n.r.d
Repeated dose toxicity (on rodents)		90-day	14-91 day	90-day	Yg	90-day
Mutagenicity	In-vitro bacteriological test	Y	n.r.b	Y	c)	Yd
	In-vitro non-bacteriological test	Y	n.r.b	Y		Yd
Exemptions		n.sp.	Possible	e)	f)	n.i.

Abbrev.: n.i. (not investigated), n.r. (not required), n.sp. (not specified), Y (required).

a) Either acute or repeated dose tests suggested on a case-by-case basis, depending largely on the species of the host organism and consideration of the nature of toxins that could theoretically be present (e.g., mycotoxin or bacterial enterotoxin). According to PARIZA & JOHNSON (2001) bacterial toxins are acute toxins and are produced by a few species only.

b) Relevant only in case of new enzyme types.

c) Depending on the outcome of the repeated dose study additional tests may be required.

d) Not explicitly required but generally accepted in recent enzyme evaluations.

e) If the production microorganism has a long history of safety in food use, and belongs to a species where no toxins are produced, and the particular strain is of well documented origin acceptance of enzyme preparation without specific toxicological testing may be justified. In case of non-toxin producing GMO: If high purity and specificity of the enzyme product could be demonstrated full toxicity testing may not be needed.

f) If both the GMM and the protein do have a history of safe consumption by humans and animals, specific toxicity testing might not be required.

g) Not specified.

The recent EFSA guidance on GMM and derived products (EFSA 2006b)²⁹ establish detailed information requirements for characterisation of the enzyme protein similar to those proposed by FDA including amino acid sequence and post-translational modifications.

Toxicity relevant information would include homology comparisons to proteins known to cause adverse effects, information on stability of the protein under processing and storage conditions and the expected treatment of the food and testing for resistance to proteolytic enzymes. Toxicity testing would include a repeated dose oral toxicity study – additional studies would depend on the outcome of the animal study. Exemptions from toxicity testing might be justified if both the GMM and the protein do have a history of safe consumptions by humans.

3.1.7 Exposure assessment and risk characterisation

As there is little specific guidance about exposure requirements in SCF and JECFA guidance on food enzymes assessment for food enzymes can be best interfered from recent JECFA practice.

Exposure assessment and risk characterisation seems to follow a kind of standard procedure described in the following and based on the examples analysed in Table 15.

²⁹ This guidance is however, not laid out for industrial enzymes and certain enzyme specific information requirements, such as for additives in the enzyme preparation, hygiene (total viable counts, production strain), contaminants including thresholds (heavy metals, microbes not included, antibiotic activity in enzyme preparation, presence of mycotoxins) are not included. Hence, it is likely that for food enzymes additional guidance will have to be provided by EFSA.

Dietary intake

Based on the individual application of the enzyme dietary intake is estimated based on the assumption that all enzyme (including all impurities from the fermentation process) applied in food processing will remain in the final food product and that the remaining enzyme (and the impurities) will be fully active. This represents a worst case assumption as most food enzymes are used as processing aids during food processing as opposed to food additives which are intended to remain in the food product. More realistically, subsequent steps in food processing, such as heat treatment or shifts in pH, are likely to remove and/or inactivate enzymes. Based on these estimates of the concentration of enzymes in final food products a cumulative dietary intake is estimated. This intake is then compared with the NOEL derived from the 90-day subchronic feeding studies in rodents to calculate the margin of safety. Normally, there is no indication of toxicity at the highest doses applied. The highest dose is therefore considered to be the NOEL. In fact, this is the minimum NOEL. Given that no indications of toxicity had been observed at the highest dose, the real NOEL could be higher.

Table 15: Exposure assessment and risk characterisation for food enzymes in JECFA.

Enzyme	Exposure relevant information	Exposure assessment	Risk characterisation
α -amylase	Enzyme is thermostable and active at low pH Absence of enzyme in the purified syrup confirmed by testing; not expected to be present in beer Enzyme is non toxic at the highest dose applied in the 90-day subchronic toxicity studies (=NOEL)	Sugar and sweeteners: i.e. 12 mg of TOS/day (a). Beer: i.e. 3 mg of TOS/day Alcohol in spirits: 0.18 mg of TOS/kg b/day Estimated cumulative intake: 0.43 mg TOS/kg bw/day	NOEL: 1.02 g TOS/kg bw/day MoS: > 2000 ADI: not specified
Xylanase	Assumption: Enzyme is described to be inactivated during bread making (no tested) Enzyme is non toxic at the highest dose applied in the 90-day subchronic toxicity studies (=NOEL)	All baking products are produced with the enzyme at the maximum recommended dosage (16 g/100 kg flour) and using a standard recipe by which 100 kg flour will produce 140 kg bread Based on upper physiological intake of 50 g/kg bw/day a maximum intake of baking products 25 g/kg bw/day was assumed leading to an intake of 0.115 mg TOS/kg bw/day, i.e. 6.9 mg TOS/day/person (a).	NOEL: 1.1 g TOS/kg bw per day MoS: 10000. ADI: not specified
Mixed Xylanase, β -Glucanase	Assumption: Beer filtration process is likely to remove the denatured enzymes (not tested) No enzymes or other organic solids are expected to be carried over into the final product because ethanol is removed by distillation from the fermentation mash containing the enzyme preparation Enzyme is non toxic at the highest dose applied in the 90-day subchronic toxicity studies (=NOEL)	All beers are produced using the enzyme mixture as a processing aid at the recommended dosage (200 g/ton barley and/or malt); one ton of barley and/or malt will produce 6000 l of beer. Estimated daily intake: Beer 5.1 mg of TOS/day (equivalent to 0.085 mg/kg bw/day) Spirit: 2.7 mg of TOS/day (equivalent to 0.04 mg/kg bw/day/person)	NOEL: 0.62 g TOS/kg bw/day MoS: 5000 ADI: not specified

ADI: Acceptable daily intake; MoS: margin of safety; TOS: total organic solids from fermentation; mainly protein and carbohydrate components

a) for a person of 60 kg

Source: amylase: <http://www.inchem.org/documents/jecfa/jecmono/v52je02.htm#die>; xylanase: <http://www.inchem.org/documents/jecfa/jecmono/v52je11.htm>; mixed xylanase, β -glucanase preparation: <http://www.inchem.org/documents/jecfa/jecmono/v52je07.htm>

Risk characterisation

The NOAEL is then divided by the estimated intake to yield the margin of safety. In JECFA assessments conducted so far the MoS ranged between 100 and 10000. Given the absence of toxicity in the animal studies and the large MoS there is no reason for safety concerns. In many cases it cannot be expected that all enzyme will remain and

be active in the final product. Furthermore, the NOAEL might be higher, thus, the effective MoS is likely to be higher in many cases.

3.1.8 Comparison of toxicity assessment requirements for GMM and food additives

Table 11 (in the Supplement Volume) provides a comparison of the SCF guidance on food enzymes (SCF 1992), the SCF guidance on food additives in general (SCF 2001), the EFSA guidance on GMM and derived products for food and feed (EFSA 2006b), and CODEX ALIMENTARIUS COMMISSION (2003) on GMM and derived products.

With the exception of the SCF (2001) Guidance all guidance documents were either exclusively (SCF 1992) aiming at proteins produced from microbes and used for food purposes or are at least focussing on proteins from microbes. The EFSA and the Codex Alimentarius Guidance are fairly similar – only the EFSA Guidance provides much more details compared to rather general recommendations included in the Codex Guidance. In general the EFSA Guidance asks for more detailed information on the protein.

History of safe consumption is a key concept in EFSA and SCF documents and can lead to reduced toxicity text packages (SCF 1992, 2001) or even to waiver from toxicity testing at all (EFSA 2006b). None of the guidance documents can be interpreted to require animal toxicity tests in any case.

EFSA (2006b) proposes a repeated dose study (appropriate duration would be decided on a case-by-case basis) in rodents and would ask for additional studies depending on the outcome of the animal test. The SCF (1992) asks for a 90-day subchronic study. This contrasts the extensive standard food additive test package comprising of 14–28-day studies, at least two 90-day subchronic studies (rodent and non-rodent), lifespan studies in rat and for chronic toxicity and carcinogenicity, genotoxicity studies, reproduction and developmental toxicity studies. Only the SCF Guidance ask for genotoxicity studies as well.

The more recent guidance documents require both toxicity tests to be conducted according to OECD/EC Guidelines and under GLP conditions.

Only the SCF Guidance is mentioning the possibility to assign a NOAEL.

The SCF and the Codex Guidance (SCF 2001, CODEX 2003) both require information on present and anticipated exposure, potential impact of dietary intake and effects on population sub-groups. The concentration of the protein in the final food should be determined according to SCF (1992) and Codex. Stability relevant information is required in all four guidance documents the details of which are varying. Most important factors seem to be heat and processing conditions. EFSA asks for storage stability as well. SCF (1992) is considering reaction products in case they are not normal constituents of the diet, EFSA is particularly asking for modifications of the protein and stable protein fragments.

Digestibility testes (in-vitro) are asked for in both the EFSA and Codex Guidance. EFSA also requires to characterise stable breakdown products.

In case of EFSA Guidance the overall approach is explicitly case-by-case based, depending on source, function/activity and history of consumption and also guided by comparison to conventional counterparts (apparently including both the micro-organism and the food/substance). The latter principle is also endorsed in the Codex Guidance. In

contrast the SCF Guidance seems to be less flexible with especially the SCF Guidance (2001) asking for a battery of core tests in any case (see above).

3.1.9 Toxicity data on food enzymes

The vast majority of toxicity assessments on food enzymes and other proteinaceous food additives have been conducted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by the FDA.

So far JECFA has evaluated about 70 assessments³⁰. More recent applications to JECFA typically contain a 14-day range finding toxicity study, a 90-day subchronic toxicity study, and two studies on genotoxicity (reverse mutation and chromosomal aberration).

The FDA evaluated more than 50 enzymes and typically includes a subchronic oral toxicity study in rodents along with studies on genotoxicity.

More than 30 assessments of food and/or feed enzymes conducted for regulatory purposes have also been published in the scientific literature and have been analysed in more detail (Supplement Volume).

Endpoints most frequently considered in a declining order are genotoxicity, oral subchronic (usually 90-day), acute oral, and subacute oral toxicity. Less frequently skin irritation, eye irritation and teratogenicity studies were conducted. Only in one case a chronic toxicity study has been described. The majority of these tests were conducted according to OECD and/or US FDA or US EPA norms. GLP compliance was explicitly mentioned in about half of the cases.

Other endpoints occasionally considered include: pathogenicity, acute inhalation toxicity, acute dermal toxicity, skin irritation.

Significant differences to the controls were occasionally detected but in none of the cases concluded to be toxicologically relevant.

PARIZA & JOHNSON (2001) evaluated 102 bacterial mutagenesis tests and 63 chromosome aberration tests of enzymes and in none of the cases did find the enzyme to be genotoxic.

The largest database on enzyme toxicity tests is existing in industry. The European Association of Manufacturers and Formulators of Enzyme Products (AMFEP) reported more than 800 toxicity tests on enzymes³¹ (including enzymes used for other purposes than food). Allegedly, none of these tests did identify a toxic enzyme protein (see Table 16).

³⁰ A list of JECFA evaluations publicly available (status: 2001).

³¹ Status 2001.

Table 16: Toxicity tests on enzymes.

Toxicity endpoint	Number of tests			
	Food	Feed	Technical	All (b, c)
Enzyme application				
Acute oral toxicity	16	2	3	119
Subacute oral toxicity	11	1	1	99
Oral subchronic toxicity	17	2	1	180
Oral chronic toxicity	1	0	0	22
Mutagenicity (bacterial)	25	2	3	199
Mutagenicity (mammalian)				172
Teratogenicity	4	0	0	N.sp.
Skin irritation	7	2	2	N.sp.
Eye irritation	7	2	3	N.sp.
Other (a)	18	2	3	N.sp.
Total enzymes assessed	> 30			> 180

a) Acute inhalation toxicity study, skin sensitization study, acute dermal toxicity study, delayed contact hypersensitivity study, antibiotic production, pathogenicity study, tolerance study.

b) Including enzymes for all applications.

c) Last column based on FEDERAL ENVIRONMENT AGENCY & IFZ (2002); acute: studies with a single oral dose given; subacute: studies with daily oral doses given (more than 1 day, usually 28 days); subchronic: studies with daily oral doses given (more than 28 days, usually 91 days); chronic: studies with daily oral doses given (more than 91 days); mutagenicity (bacterial): in-vitro mutation studies using bacterial cells; mutagenicity (mammalian): in-vitro mutation studies using mammalian cells.

N.sp.: Not specified.

As the vast majority of these tests are not publicly available this is difficult to verify. Granted, that essentially all these toxicity tests have failed to detect a toxic enzyme protein one might be tempted to draw general conclusions about the absence of toxic properties of enzymes or even about proteins. In the view of the authors of this report this would be premature for a number of reasons discussed in Chapter 4.

3.2 Biopesticides

This Section describes the assessment of toxic properties for mammals of microbial biopesticides. Proteinic biopesticides expressed in GM plants (e.g. **Bt**-plants) are not covered in this Section. Given the easier accessibility of regulatory documents the focus lies on the USA. Section 3.2.1 briefly describes technical aspects of the main types of microbial and plant-derived biopesticides. Section 3.2.2 summarizes the lesson drawn from toxicity assessments and other studies on **Bt** products. Section 3.2.3 describes the US EPA regulatory approach to toxicity assessment. A brief description of the EU system is given in Section 3.2.3.2.

Biopesticides as a category encompass microbial pesticides and biochemicals derived from microorganisms and plants as well as plant-incorporated protectants (PIPs). PIPs is a term used in the USA for pesticidal substances expressed by GM plants and are covered elsewhere in this report. Biopesticides include well known microbial pesticides with a long history of use such as **Bacillus thuringiensis** as well as recently developed substances from other bacterial and fungal species (SUDAKIN 2003).

Microbes as biopesticides represent a fast growing niche in the worldwide pesticide business. Their increasing application poses challenges for clinical toxicologists. In contrast to classical pesticides they expand the scope of plausible health risks to include infectious and immunological mechanisms. The incidence of human health effects is

very rare, but the complexity of the organisms makes a profound understanding of their microbiology, ecology, target specificity and the pathogenic as well as immunological properties more difficult.

3.2.1 Types of biopesticides

3.2.1.1 *Bacillus thuringiensis* (*Bt*) products)

Bt represents the most widely used type of biopesticides. *Bt* is a gram-positive, motile, rod-shaped, endospore-forming bacterium which is ubiquitous, found in soils world-wide. During the sporulation process δ -endotoxin is produced, which is a crystal protein (Cry protein) and the pre-form of the active ingredient in *Bt* products. The biopesticide can be applied by several methods including hand spraying, aerial spraying, and ground based applications. It can also be applied through irrigation systems. The first microbial *Bt*-product commercialized in 1961 was based on *Bt kurstaki* strain HD1. The efficacy of the HD1 strain largely results from the presence of four Cry proteins Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa. Cry proteins belong to a class of bacterial toxins known as pore-forming toxins (PFT), which are secreted as water soluble proteins undergoing conformational changes in order to insert into, or translocate across, cell membranes of their host. *Bt* pesticides can be marketed as inactivated microorganisms, which may be the original

B. thuringiensis or other genera. GM *Pseudomonas fluorescens*, for instance, is also used to express Cry genes. There are several practical reasons for commercially producing the toxins in *P. fluorescens* rather than in the original strains of *Bt*: (i) *P. fluorescens* produces higher concentrations of the toxins than *Bt*; (ii) the Cry proteins remain within the *P. fluorescens* cells ("encapsulated") when the cells are inactivated; (iii) the inactivated *P. fluorescens* cells protect *Bt* endotoxins from decomposition induced by ultraviolet light, thereby extending the time the toxins remain active; (iiii) *P. fluorescens* does not produce the large number of additional toxins made by *Bt*, some of which might harm non-target species.³²

After the *Bt* toxin is ingested by the insect, the Cry protein is activated by cleavage and subsequently binds to mid-gut epithelial receptors leading to gut paralysis and the insect stops eating.

3.2.1.2 Other bacterial biopesticides

Beside *Bt* biopesticides there are other bacterial strains with insecticidal or antifungal activity, especially other *Bacillus* spp. as well as *Pseudomonas* spp. some examples are shown in Table 17.

Table 17: Examples of bacterial biopesticides and their mode of action.

Bacterial species	Mechanism of action
<i>Agrobacterium radiobacter</i>	Competitive inhibition of pathogenic bacterial species (<i>Agrobacterium tumefaciens</i>)
<i>Bacillus licheniformis</i> strain SB3086	Production of anti-fungal enzyme
<i>Bacillus subtilis</i> GB03	Competitive inhibition of pathogenic fungal species
<i>Pseudomonas chlorophasis</i> strain 63-28	

Source: SUDAKIN (2003)

³² *Bt* can express a β -exotoxin, a non-proteinous toxin with considerable mammalian toxicity.

3.2.1.3 Fungal biopesticides of entomopathogenic type

Certain fungi are pathogenic to insect hosts, referred to as entomopathogenic. Examples are *Metarhizium anisopliae*, which is a natural pathogen to insects and used for controlling grasshoppers. *M. anisopliae* strain ESC1 is used as a termiticide and shows no mammalian toxicity in animal tests. The main reason is that *M. anisopliae* cannot grow at mammalian body temperatures. Despite reports of immunological reactions in animal studies, there are no reports of human hypersensitivity reactions following biopesticide application.

3.2.1.4 Other types of fungal biopesticides

Myrothecium verucaria is a fungal species capable of producing a macrocyclic mycotoxin applicable to weed control. The use in weed control is being investigated. *M. verucaria* is registered in the US as nematocide applied as a heat-inactivated mixture the working mechanisms of which are not well understood. An *Aspergillus flavus* strain received US regulatory approval for application as fungicide applied to cotton for reducing aflatoxin contamination. It works by competitive inhibition of aflatoxin producing strains.

Table 18: Examples of fungal biopesticides and their mode of action.

Fungal species	Mechanism of action
<i>Aspergillus flavus</i> strain AF36	competitive inhibition of toxigenic fungal species
<i>Gliocladium catenulatum</i> strain J1446	Production of anti-fungal metabolites
<i>Metarhizium anisopliae</i> strain ESC1	Termiticidal and insecticidal, pathogenic to insects
<i>Myrothecium verrucaria</i>	Heat-killed fungus, nematocidal

Source: SUDAKIN (2003)

3.2.1.5 Research on plant derived biopesticides: lectins

It has been extensively demonstrated that plant lectins are effective biological agents against insect attack. Therefore, insect-resistant plants produced by expression of lectin genes in transgenic plants are a major goal of R & D activities. However, only a few lectins have so far been identified to convey high resistance to plant pests whilst simultaneously having a low toxicity to higher mammals, including man.

3.2.2 Toxicity data on microbial proteins used as biopesticides

This section focuses on formulations and active principles (Cry proteins) from *Bt* strains because of their long history of application, their particular relevance for organic farming, and the extensive knowledge database. *Bt* toxicity is highly specific to target insects, for instance the *Bt* subspecies *aizawai*, *kurstaki*, *israelensis* or *tenebrionis* differ in their host range. The pesticides are used for food and non-food crops, in greenhouses and forests as well as for outdoor home use. *Bt* products are marketed as granular powder, dust, suspension and in a liquid form.

3.2.2.1 Toxicity / pathogenicity testing of *Bt*- preparations

Testing of microbial *Bt*-preparations has been done to investigate acute, sub-chronic and chronic adverse effects on human health. Studies were submitted to the U.S. EPA in the course of pre-market authorization. Animal testing was performed with technical grade materials of microbial pesticides, most of them based on *Bt kurstaki*. Studies were conducted in rats as well as in humans, and NOEL were derived (see also Table 19).

Unfortunately, a range of different dose units are used making it difficult to compare the results: mg technical ingredient /kg body wt; CFUs /kg body wt; spores/kg body weight (McCLINTOCK 1995). In new studies, exposure is expressed in terms of colony forming units (CFU), while dose levels in the studies were sometimes quantified by weight of material administered. In the acute studies, dosing ranges from 10^7 to 10^8 CFU per animal.

In acute toxicity/pathogenicity studies (examples given in Table 19) no adverse effects or signs of infectivity to mammals considering mortality were observed, nor changes in body weight or gross pathology findings at necropsy. Subchronic toxicity studies in rats indicate NOELs in the range of several thousand mg **Bt** product per kg bw per day. In a 2-year chronic rat feeding study decreased weight gain in females dosed with 8400 mg/kg/day was observed but not considered to be of toxicological concern. Human volunteers have been fed 1000 mg of **Bt**-microbial preparation per day for several days - yet they showed no symptoms of toxicity or other adverse effects (BETZ et al. 2000).

Table 19: Mammalian toxicity/ pathogenicity assessment studies of *Bt*-products

<i>Bt</i> product (technical grade)	Type of study	NOEL	Findings	Reference
<i>Kurstaki</i> (Crymax) Cry1Ac, Cry2A, Cry1C	Acute oral toxicity/ pathogenicity (rat)	> 2.5–2.8 108 CFUs/rat	No evidence of toxicity	EPA (1996) CARTER & LIGETT (1994)
<i>Kurstaki</i> (Dipel) Cry1Aa, Cry1Ab; Cry1Ac, Cry2A	2 year chronic fed (rat)	8400 mg/kg/day	Statistically significantly decreased body weight gain in females from week 10 to week 104 (not considered related to Cry proteins); no infectivity/ pathogenicity was found	McCLINTOCK et al. (1995)
<i>Kurstaki</i> Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa	Human - oral	1000 mg/adult	No toxicity, infectivity; all blood cultures were negative; 5 of 10 patients show viable <i>Bt</i> microbes in stool samples 30 days post feeding	EPA (1986) McCLINTOCK et al. (1995)

Source: BETZ et al. (2000)

Additional testing for registration of **Bt** microbial products has been done by administering the preparations via non-oral routes of exposure, such as pulmonary, dermal, ocular, intraperitoneal, subcutaneous, intravenous or intracerebral to assess the pathogenicity/infectivity of the **Bt** organism. It was found that large doses (10^8 CFUs) of injected **Bt** causes occasional mortality in rodents. Since mortality can occur following injection of large doses of non-pathogenic micro-organisms, the observed effect was not attributed to Cry proteins present in the **Bt** preparations (EPA 1998a).

Despite of ubiquitous exposure to humans, reports of adverse reactions are extremely rare. Since it is used as a biopesticide, occupational health studies were conducted, for instance on greenhouse workers following contact with **Bt** aerosol. The strains used could be found in 40 % of the workers stool samples but there was no incidence of adverse gastrointestinal effects. Another study found an immunological reaction to **Bt** (skin prick test) in workers following biopesticide use, but no symptoms or signs of allergic disease were reported (SUDAKIN 2003).

In-vitro studies simulating gastric fluids showed, that Cry proteins are rapidly degraded in SGF while being resistant in SIF. These in-vitro models are deemed by some authors

to be significantly less robust than the gastrointestinal systems of humans and animals, which suggests that the Cry proteins will be rapidly degraded following consumption (BETZ et al 2000)³³. Contradicting evidence comes from experimental animal feeding study using crystal Cry1Ab protein (see Section 2.8.1). Some studies detected intact proteins or large fragments in the intestines of swine and other test animal species while other failed to detect the protein. SUDAKIN (2003) failed to find evidence that the protein was absorbed from or distributed outside of the gastrointestinal tract.

3.2.2.2 Toxicity relevant aspects

The mode of action of Cry proteins involves solubilisation of the protein crystals in the insect mid-gut, proteolytic processing of the protoxin by mid-gut proteases to yield the active toxin, binding of the active Cry toxin to mid-gut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores (SCHNEPF et al. 1998). There are very few studies in the public literature analyzing the potential for human health effects from individual Cry proteins (OECD 2007). Acute rodent studies have been performed on several Cry proteins produced from various GM and non-GM microbial sources: Cry1Ab, Cry1Ac, Cry3A, Cry1F, Cry2Ab2, Cry3Bb1. In all cases the Cry proteins was administered orally and showed no adverse effects in mice at high doses ranging from 3760 to 5220 mg/kg bw (OECD 2007).

Possible reasons for the low or lacking mammalian toxicity are (i) the requirement of an alkaline environment for the activation of the protoxin. Alkaline environments are typical for insect guts but not for mammals. (ii) the Cry1Ab protein for instance was shown to be rapidly degraded under in-vitro digestibility studies.³⁴; (iii) receptor-mediated binding to the brush border membrane in mid-gut epithelium cells leads to membrane-bound forms of the Cry protein. This is selective in insects and has not been convincingly shown in mammals, so far (NOTEBORN et al. 1993).³⁵

SJOBLAD et al. (1992) discusses toxicity evaluation of proteins used as biopesticides in its pure form:

“Proteins are susceptible to acid and enzymatic digestion to amino acids prior to assimilation. Certain proteins are known to be toxic to humans, and these have received wide study. If toxicity testing of a protein is considered necessary, the acute exposure studies in laboratory animals should be sufficient, since – if toxic – proteins are known to act via acute mechanisms.”

3.2.3 Toxicity assessment of biopesticides

3.2.3.1 U.S. EPA data requirements using the example of *Bt*-(re)registration

As of 2006 225 microbial products were authorised in the USA, 145 of them *Bt* products.³⁶ The safety testing requirements for registration of *Bt*-products have evolved over the years based on EPA reviews of completed toxicity/pathogenicity studies in 1982, in 1989, and again in 1998. Current data requirement for registration of (non-GM) conventional microbial pesticides was published in the Federal Register Title 40, Part 158 of the Code of Federal Registrations (CFR).

³³ For an extensive discussion on in-vitro digestibility testing see Section 2.8.

³⁴ For an extensive discussion on the relevance of in-vitro digestibility testing see Section 2.8.

³⁵ There have been occasional reports on Cry1Ab binding to mammalian gut surface as well as biological effects in mammals. However, the relevance of these observations is still unclear (e.g. Noteborn et al. 1995; see discussion in Section 2.4).

³⁶ Regulation of Biological Control Agents (REBECA) Workshop: Potential risks of microbials. Innsbruck 12th & 13th of April 2006

Besides proteins as active ingredient in microbial pesticides proteinic substances can also be present as by-products or contaminants from the fermentation process. *Bacillus thuringiensis* subsp. generate toxic factors, many of them proteins. The information required by the EPA for registration do not involve the isolation of protein components for evaluation of toxic effects. Instead, the potential for toxicity of protein components is determined by testing of the active microbial ingredient together with the fermentation medium in laboratory animals. In addition, EPA requires that manufacturers submit a thorough taxonomic characterization of the production strain as well as a description of the manufacturing process, including measures taken to minimize the presence of contaminating organisms.

Newly prepared batches or lots of manufactured microbial pesticides are required to be screened for the presence of certain human pathogens, and, in case of *Bt* products, by subcutaneous injection of newly prepared batches into rodents (SJOBLAD et al. 1992).

Further mammalian toxicity testing for hazard characterisation is required for microbial pesticides. Potential toxicity is determined by testing the active microbial ingredient together with the fermentation medium in laboratory animals and non-target organisms.

The data requirements are structured in a tiered testing regime.

The tiered approach aims at requiring only those studies considered necessary for an adequate human health risk evaluation. The information compiled in Tier I allows assessment for the potential of the micro-organisms to be pathogenic or toxic to mammals. However, if toxicity is observed in test animals, in the absence of signs of pathogenicity, then the toxic components in the test material are to be identified and, if possible also, isolated. Generally, if significant adverse effects are observed in Tier I, a Tier II study may be needed.

Table 20: Mammalian toxicology data requirement according to 40 CFR 158.740.

Tier 1 studies on the active microbial ingredient Acute toxicity/pathogenicity³⁷ testing (oral, dermal, pulmonary and iv) on rat/mouse Primary eye irritation on rabbit Reporting of hypersensitivity incidents (Mammalian cell culture studies with viral pest control agents)
Tier 2 studies Acute toxicity Subchronic toxicity/pathogenicity
Tier 3 studies (chronic exposure) Reproductive and fertility effects Oncogenicity Immunodeficiency Primary infectivity /pathogenicity

Source: McCLINTOCK (1998)

In the acute toxicity/ pathogenicity Tier I studies, the mouse or the rat is exposed to a single high dose of micro-organisms. Three studies are reflecting three routes of exposure: oral, pulmonary and intravenous (or intraperitoneal). Test animals are evaluated by determining mortality, body weight gain, clinical signs of toxicity (gross necropsy) and evaluating the pattern of clearance of the micro-organism from the animals.

Pathogenicity: From appropriate organs, tissue and body fluids of test animals microorganisms are periodically enumerated to ensure lack of infectivity or persistence in mammals.

“The information from these acute toxicity studies allows an assessment for the potential of the micro-organism to be pathogenic, or toxic, to mammals. In most cases, a lack of adverse effects allows for the reasonable conclusion that the protein component of the micro-organism are not toxic to mammals [...] The potential toxicity of proteins in the growth or fermentation medium can be evaluated by including the growth/fermentation materials in the dosing material for the oral, pulmonary, or intraperitoneal studies” (SJOBLAD 1992).

For non-target organisms (terrestrial and aquatic invertebrates and plants) an analogous tiered approach is provided.

For biopesticides produced from genetically modified micro-organisms, the health-risk assessment process is not significantly different from non-GM ones.

In 1998 a re-registration³⁸ for **Bt** formulations was conducted with the results being publicly available (EPA 1998a)³⁹. Referring to the applied database an explicit reference is made to a review article of McCLINTOCK et al. (1995). According to the human health assessment the document state, that “to date, no known mammalian health effects have been demonstrated in any infectivity/pathogenicity study. Some strains of *Bacillus thuringiensis* have the potential to produce various toxins that may exhibit toxic symptoms in mammals, however the manufacturing process includes monitoring to prevent these toxins from appearing in products” (EPA 1998a). The report further states, that the overall toxicity studies are sufficient to waive studies demanded under Code of the Federal Regulations Title 40, Part 158 (40 CFR Part 158): “The sum total of all toxicology data submitted to the Agency [...] allows the conclusion that the infectivity/pathogenicity studies normally required under 40 Code of the Federal Regulations, Part 158, for the use patterns of the registered products be waived in the future as long as product identity and manufacturing process testing data indicated there is no mammalian toxicity associated with the strain” (EPA 1998a: 11). Given the lack of adverse effects it is concluded that protein components of the micro-organism are not toxic to mammals (McCLINTOCK 1998).

³⁸ Re-registration case 0247, *Bacillus thuringiensis*. Search and download under <http://www.epa.gov/nscep/>.

³⁹ The results are documented in a Re-registration Eligibility Decision document (EPA 1998a) and an accompanying summary document (EPA 1998b).

Table 21: Excerpt from Eligibility Decision document.

Data Supporting Guideline Requirements for the Reregistration of <i>Bacillis thuringiensis</i>				
REQUIREMENT		USE PATTERN	CITATION(S)	
885.1500	Certification of Limits/Analytical Methods	All	41439002, 41980101, 41429703, 41435402, 41751104, 42015901, 41789701, 41653901, 41657002, 41646702, 41429603	
885.1600	Physical & Chemical Limits	All	41439002, 41503903, 41429704, 41435402, 42080102, 42015901, 41429704, 41653901, 41503902, 41503904, 41429604	
TIER I TOXICOLOGY				
885.3050	Acute Oral Toxicity/Pathogenicity	All	waived ¹	
885.3150	Acute Pulmonary Toxicity/Pathogenicity	All	waived ¹	
885.3200	Acute Intravenous - Toxicity/Pathogenjcity	All	waived ¹	
885.3400	Hypersensitivity Incidents	All	42027101	

Source: EPA Document (EPA 1998a)

Table 22: Acute mammalian toxicity for *Bt*. Excerpt from Eligibility Decision document.

Guideline Numbers*	Study	Results	Toxicity Category	MRIDs	
152A-10 (885.3050)	Acute Oral Toxicity/Pathogenicity	No adverse toxic effects, infectivity, or pathogenicity seen at doses up to 4.7×10^{11} spores/kg.	IV	142733 41046704 42006502 43186101 40951102	96520 96527 96533 109492 246968
152A-12 (885.3200)	Acute Pulmonary Toxicity/Pathogenicity	No adverse toxic effects, infectivity, or pathogenicity seen at doses up to 2.6×10^7 spores/kg.	IV	96529 41308603 42006503	
N/A (generally received under 151A-10, Product Analysis)	Acute Intraperitoneal Toxicity/Pathogenicity	Non toxic at dose levels below 10^6 colony forming units (CFU) per animal. No infectivity or pathogenicity.	N/A	66178 66179 90207 90208 41590302 41270301 41308607 41441504 41441505 41441506	41441609 41441611 41441612 41722507 41826608 41826609 41994303 42750401 42791301
152A-15 (885.3400)	Hypersensitivity Incidence Reporting	Two possible incidences reported, neither one was caused by <i>Bacillus thuringiensis</i> .	N/A	420271	
81-2 (870.1200)	Acute Dermal Toxicity	No dermal toxicity observed at doses up to 4.7×10^{11}	IV	142734 109493 404974	419943 41412705

* 1988 Subdivision M (1995 Harmonized Guidelines)

Source: EPA (1998a), Appendix B, p 59.

EPA proposal for data requirements for microbial pesticides in the Federal Register Part 158 (EPA 2006) and FIFRA background document (FIFRA 2000)
In the EPA proposal update and revise of the data requirements for the registration of microbial pesticide products is made to reflect current scientific knowledge. The

proposal for amending Part §158.950 (human health assessment data requirement) continues to follow the tiered approach: In practice, this means that acute toxicity testing of manufacturing – use product (MP) or the end-use product (EP) is seen as principally sufficient (= required data) to evaluate the pesticide product. Further repeated dose testing, e.g. testing on subchronic toxicity, reproductive fertility effects, carcinogenicity, immunotoxicity) is condition depended. A FIFRA document (FIFRA 2000) outlines and constitutes the fundamental EPA position about the relevance or necessity of long term or repeated dose animal testing in relation to acute testing on biopesticides.⁴⁰ It is subsequently cited:

“One of the features of current biopesticide data requirements that differs significantly from the requirements for conventional pesticides, is the tiering or stepwise progression of the data needed for addressing mammalian hazard identification. Acute and subchronic (90-day) testing is routinely required in the initial tier of testing for conventional pesticides in order to evaluate risk and identify potential target organs to focus on in the required chronic (18–24 months) tests. The situation is somewhat different for biopesticides, which include pesticides using either microorganisms or biochemicals. Subchronic (90-day) testing for biochemical pesticides, which are naturally occurring compounds with a non-toxic mode of action, can be required if residues of the compound are expected to be present in significant amounts in treated food or animal feed. For microbial pesticides, the data requirements are all based on acute, single dose, maximum hazard exposures. However, these single dose exposures are specifically designed to examine pathogenicity and infectivity in addition to toxicity. If toxicity from the microbial pesticide is observed in these acute tests, further testing to classify the toxicity or longer term, repeat dose testing would be triggered. EPA believes that an approach similar to biopesticides, especially microbial pesticides, is justified for examining protein plant-pesticides. The majority of the exposure should be in the diet¹. The most protective approach for examining these dietary exposures is to test the purified protein at high doses in an acute test. If the protein has been shown to be structurally (by amino acid sequence homology) or functionally related to a protein known to have longer-term effects in mammalian species, then longer term tests may be justified. Examples of the types of proteins known to have longer-term effects in mammals would be those that inhibit digestive enzymes or that bind essential nutrients so that they are not available.

For the most part, proteins that would be expected to be toxic should express toxicity when tested at the high doses required in the acute oral test. The results of repeat dose (30-day) toxicity tests performed to date for both tomato expressing Cry1Ab toxin and corn expressing the Cry9C toxin have confirmed that lack of toxicity in the single high dose acute test indicates that repeated dose studies will also be negative for toxicity (Noteborn et al., 1995; and the data evaluation report available at <http://www.epa.gov/pesticides/biopesticides/cry9c/der-44734303a.htm>). Therefore, if the results of the acute test were negative and no structural relationship was seen with toxins, allergens or other proteins that may have known longer-term toxicity, then no further testing would necessarily be indicated. However, if a protein plant-pesticide were suspected of having a longer-term effect, either by the results of the acute test or by relationship to previously identified, longer-term toxins, the protein would require follow-up in a repeat dose (30 day) testing to verify their safety. EPA believes that the longer term test, if required, should be performed with a diet that properly nourishes the test animal yet contains sufficient amounts of the protein plant pesticide test substance to provide adequate safety margins. However, EPA believes that safety testing using whole food has severe limitations. One limitation would be the inability to establish a margin of safety for the results given the low level of protein expression in plant tissue. Another limitation would be the lack of appropriateness of the plant tissue

⁴⁰ EPA is responsible for determining that plant pesticides can be safely used under the Federal Fungicide, Insecticide and Rodenticide Act (FIFRA).

as laboratory animal food. It is possible that unforeseen toxicity could result from the plant tissue itself being an unusual component for the test rodent in these studies. The stipulation of using the test substance itself for the repeat dose dietary tests would be a critical factor as production of adequate amounts of pure test substance for these tests bears considerable cost. Any other toxicity tests that may be needed to determine the safety of these products will be developed on a case by case basis.”

Table 23: Excerpt from EPA proposal.

(d) *Test notes.* The following test note is applicable to the data requirements for microbial residue as referenced in the last column of the table contained in paragraph (c) of this section.

1. Required when the results of testing:

i. Indicate the potential to cause adverse human health effects or the product characterization indicates the microbial pesticide has a significant potential to produce a mammalian toxin; and ii. The use pattern is such that residues may be present in or on food or feed crops.

§ 158.1040 Toxicology data requirements table.

(a) *General.* Sections 158.100 through 158.130 describe how to use this table to determine the toxicology data requirements for a particular pesticide product. Notes that apply to an individual test and include specific conditions, qualifications, or exceptions to the designated test are listed in paragraph (e) of this section.

(b) *Use patterns.* (1) This category includes products classified under the following general uses: terrestrial food and nonfood crop use; terrestrial feed crop use; aquatic food and nonfood crop use; greenhouse food and nonfood crop use; forestry; residential outdoor and indoor; and indoor food use.

(2) Nonfood use patterns include products classified under the general use patterns of terrestrial nonfood crop use; aquatic nonfood residential use; aquatic nonfood outdoor use; aquatic nonfood industrial use; greenhouse nonfood crop use; forestry use; residential outdoor use; residential indoor use; indoor food use; indoor nonfood use.

(c) *Key.* R=Required; [R]=Required for registrations and experimental use permits; CR=Conditionally required; [CR]=Conditionally required for registrations and experimental use permits; NR=Not required; MP=Manufacturing-use product; EP=End-use product; TEP=Typical end-use product; TGA=Technical grade of the active ingredient; All= all of the above. Specific conditions, qualifications, or exceptions to the designated test procedures appear in paragraph (e) of this section, and apply to the individual tests in the following table:

(d) *Table.* The following table shows the data requirements for microbial toxicology. The test notes are shown in paragraph (e) of this section.

TABLE—MICROBIAL TOXICOLOGY DATA REQUIREMENTS

Guideline Number	Data Requirement	All Use patterns	Test substance	Test notes
Tier I				
885.3050	Acute oral toxicity/pathogenicity	[R]	TGA	1
885.3150	Acute pulmonary toxicity/pathogenicity	[R]	TGA	—
885.3200	Acute injection toxicity/pathogenicity/(intravenous) Acute injection toxicity/pathogenicity/(intraperitoneal)	[R]	TGA	2
885.3400	Hypersensitivity incidents	[R]	All	3
885.3500	Cell culture	[R]	TGA	4
870.1100	Acute oral toxicity	[R]	MP , EP	1,5
870.1200	Acute dermal toxicity	[R]	MP , EP	5
870.1300	Acute inhalation toxicity	[R]	MP , EP	5,7
870.2400	Acute eye irritation	[R]	MP , EP	5
870.2500	Primary dermal irritation	[CR]	MP , EP	5,6

TABLE—MICROBIAL TOXICOLOGY DATA REQUIREMENTS—Continued

Guideline Number	Data Requirement	All Use patterns	Test substance	Test notes
Tier II				
885.3550	Acute toxicology	CR	TGAI	8
885.3600	Subchronic toxicity/pathogenicity	CR	TGAI	9
Tier III				
885.3650	Reproductive fertility effects	CR	TGAI	10,14
870.4200	Carcinogenicity	CR	TGAI	11,14
870.7800	Immunotoxicity	CR	TGAI	12,14
885.3000	Infectivity/pathogenicity analysis	CR	TGAI	13,14

(e) *Test notes.* The following test notes are applicable to the data requirements for microbial toxicology as referenced in the last column of the table contained in paragraph (d) of this section:

1. The acute oral toxicity/pathogenicity study is required to support the TGAI. However, it can be combined with the unit dose portion of the acute oral toxicity study, with an EP or MP test material to fulfill the requirement for the TGAI and the MP or EP in a single study, if the new protocol is designed to address the endpoints of concern.

2. Data not required for products whose active ingredient is a virus. For test materials whose size or consistency may prevent use of an i.v. injection, the i.p. injection procedure may be employed.

3. Hypersensitivity incidents for registered products must be reported if they occur.

4. Data must be submitted only for products whose active ingredient is a virus.

5. The 870 series studies for the MP and EP are intended to provide data on the acute toxicity of the product. Waivers for any or all of these studies may be granted when the applicant can demonstrate that the combination of inert ingredients is not likely to pose any significant human health risks. Where appropriate, the limit dose approach to testing is recommended.

6. Data are required only if dermal irritation is found after dosing in acute dermal toxicity study.

7. Required when the product consists of, or under conditions of use would result in, an inhalable material (e.g., gas, volatile substances, or aerosol particulate).

8. Data required when significant toxicity, in the absence of pathogenicity and significant infectivity, is observed in acute oral, injection, or pulmonary studies (Tier I). Route(s) of exposure correspond to routes where toxicity was observed in Tier I studies. The toxic component of the TGAI is to be tested.

9. Data required when significant infectivity and/or unusual persistence is observed in the absence of pathogenicity or

toxicity in Tier I studies. Routes of exposure (oral and/or pulmonary) correspond to routes in Tier I studies where adverse effects were noted. Data may also be required to evaluate adverse effects due to microbial contaminants or to toxic byproducts.

10. Data are required when any of the following criteria are met:

(i) Significant infectivity of the microbial pest control agent (MPCA) was observed in test animals in the Tier II subchronic study and in which no significant signs of toxicity or pathogenicity were observed.

(ii) The microbial pesticide is a virus which can persist or replicate in mammalian cell culture lines.

(iii) The microbial pesticide is not amenable to thorough taxonomic classification, and is related to organisms known to be parasitic for mammalian cells.

(iv) The microbial pesticide preparation is not well purified, and may contain contaminants which are parasitic for mammals.

11. Data may be required for products known to contain or suspected to contain carcinogenic viruses or for microbial components that are identified as having significant toxicity in Tier II testing.

12. Data may be required for products known to contain or suspected to contain viruses that can interact in an adverse manner with components of mammalian immune system.

13. An analysis of human infectivity/pathogenicity potential using scientific literature, genomic analysis, and/or actual specific cell culture/animal data may be required for products known to contain or suspected of containing intracellular parasites of mammalian cells for products that exhibit pathogenic characteristics in Tier I and/or Tier II, for products which are closely related to known human pathogens based on the Product Analysis data, or for known human pathogens that have been "disarmed" or rendered non-pathogenic for humans.

14. Test standards may have to be modified depending on the characteristics of the microorganism. Requirements may vary for

these studies depending on the active ingredient being tested. Consultation with the Agency is advised before performing these Tier III studies.

§ 158.1050 Nontarget organisms and environmental fate data requirements table.

(a) *General.* Sections 158.100 through 158.130 describe how to use this table to determine the terrestrial and aquatic nontarget organisms data requirements for a particular microbial pesticide product. Notes that apply to an individual test including specific conditions, qualifications, or exceptions to the designated test are listed in paragraph (e) of this section.

(b) *Use patterns.* Aquatic uses include: food and feed, nonfood uses (e.g., outdoor, residential, and industrial). Terrestrial uses include: Food, Feed, Non-Food, Forestry, Residential outdoor, greenhouse (food and food), Indoor (food and nonfood), and Industrial.

(c) *Key.* R=Required; [R]=Required for registrations and experimental use permits; CR=Conditionally required; [CR]=Conditionally required for registrations and experimental use permits; NR=Not required; MP=Manufacturing-use product; EP=End-use product; TEP=Typical end-use product; TGAI=Technical grade of the active ingredient; All= all of the above. Specific conditions, qualifications, or exceptions to the designated test procedures appear in paragraph (e) of this section, and apply to the individual tests in the following table:

(d) *Table.* The following table shows the data requirements for microbial nontarget organisms and environmental fate. The test notes are shown in paragraph (e) of this section.

Source: EPA (2006)

3.2.3.2 State of regulation and data requirements in the EU

In the EU microbial pesticides are regulated by EU directive 91/414/EEC on plant protection products. Annex I to the Directive includes a positive list of active substances. Member States can only authorise the marketing and use of plant protection products based on the active substances listed in Annex I. As of 2006 57 microbial products are authorised in the EU.

For inclusion into the positive list applicants have to submit dossiers according to Annex II and III of the Directive. An assessment report is drafted by a rapporteur Member State in agreement with EFSA. Data and evaluation are discussed by the Member States and eventually a vote will decide on inclusion into Annex I. It is affirmed that the EU evaluation process works very slowly, ascribing this to a lack of resources and diverging evaluation and interpretation of data.⁴¹

Data requirements on human health effects as defined in Annex IIB are similar to those of the US EPA and also following a tiered approach. In the absence of EU guidelines the US guidelines are being used. Different to the USA 28-day toxicity tests are required in the EU but not provided in each case. In exceptional cases a special study design is agreed between the applicant and the authorities. At present there is an ongoing discussion about possible waiving of testing requirements.⁴²

A European Commission list (SANCO 3010 rev 09/08/2007) provides a topical overview with regard to the review programme for existing active substances. According to that document all *Bt* species (subsp. *aizawai*, *israelensis*, *kurstaki* and *tenebrionis*) are included in priority list 4C, for which a re-evaluation is pending and decision about authorisation and uptake into Annex I can be expected at the end of 2008.

3.3 Summary

Food enzymes

Food enzymes are the only category of single compounds of proteinaceous nature routinely used in food or food processing. Food enzymes are subjected to a safety evaluation including toxicity assessment prior to market commercialisation. Safety evaluation is mandatory for food enzymes used as food additives in the EU and for all food enzymes which are not recognised as GRAS in the USA. Proposed EU regulations will cover all food enzymes regardless of its use. Both type of food enzymes are assessed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Guidance documents are available from the former EU Scientific Committee on Food (SCF), JECFA, the FDA and the European Association of Manufacturers and Formulators of Enzyme Products (AMFEP). Furthermore, guidance documents on genetically modified micro-organisms (GMM) and derived food and feed products (EFSA, Codex Alimentarius Commission) as well as on food additives are relevant.

Guidance requirements are quite similar with respect to basic technical data on the enzyme, as the active compound, and on the source organism. Additional information is usually required in case of GMMs. Most detailed information characterising the enzyme is required by the FDA covering enzymatic function, mode of action, substrate specificity, molecular weight, isoelectric point, kinetic properties, specific activity, temperature, pH, and inorganic ions. The particular requirements for toxicological testing are primarily depending on the nature of the microbial source, e.g. if it is a microbe that also naturally occurs in food, if it is a GMM etc. toxicity endpoints suggested in guidance documents are fairly similar. History of safe consumption is a key concept in EFSA and SCF documents and can lead to reduced toxicity test packages (SCF) or even to waiver from toxicity testing at all (EFSA).

SCF and JECFA are asking for a 90-day subchronic toxicity test along with mutagenicity tests on bacteria and an in-vitro test for chromosomal aberration, while others are not specifying the length of the feeding study (FDA, AMFEP, EFSA, Codex Alimentarius Commission). PARIZA & JOHNSON (2001) proposed acute toxicity tests e.g. for enzymes

⁴¹ Fjelsted, A. (2006): Current regulation system and data requirements within the EU. Potential risks of microbials (REBECA Workshop. Innsbruck 12th & 13th of April 2006.

⁴² Expert interview 2007

from bacteria known to produce acute toxins only and to discontinue genotoxicity testing. Nevertheless, the practice described above continues.

In-vitro digestibility tests and homology comparisons to known toxins are required in the GMM specific guidance by EFSA and Codex only.

Exposure assessment as accepted by JECFA usually is based on a worst-case scenario assuming that the total amount of food enzyme used in food/food production according to manufacturers instructions will remain active in the final food product. Risk characterisation is done by calculating a margin of safety (MoS) based on the NOEL derived from the 90-day studies and the estimated dietary intake. The MoS usually is in the range of 100 to 10000. Acceptable daily intakes (ADIs) are not specified.

Toxicological data exists from regulatory evaluations of toxicity tests: about 70 enzymes have so far been assessed by JECFA, and some 50 enzymes by the FDA. Given the absence of harmonised EU legislation on most food enzymes EU Committees very rarely ever evaluated food enzymes. Toxicity testing for more than 30 food and feed enzymes are described in the scientific literature. The by far largest database on toxicity testing exists within industry (AMFEP member companies), comprising of more than 800 tests conducted on at least 180 different food enzymes. So far, publicly available toxicity test did not suggest any toxic properties of food enzymes. Industry claims that this is true for all food enzymes tested so far.

Biopesticides

Biopesticides can be based on pheromones, plant extracts or semichemicals, but only those containing of or derived from microorganisms are considered in this chapter. From an application perspective, *Bacillus thuringiensis* (**Bt**) is the most widely used biologically produced pest control agent. In 1995 it represented 2 % of the total global insecticide market. As of early 1998, there were about 200 registered **Bt** products available in the US. Cry proteins are the active principle in **Bt** and are highly specific to certain insects.

Other microorganisms are used as alternative delivery systems, e.g. *Pseudomonas fluorescens* with the benefit of higher toxin concentrations and better encapsulation.

Another major class of microbial pesticides includes insect pathogenic fungi. For instance, the use of *Myrothecium verucarria* in weed control is subject of ongoing research.

With respect to toxicity assessment the focus of this Chapter is more on US regulations because of a longer regulatory history for biopesticides based on micro-organism compared to the EU. Given its relative importance in among microbial biopesticides the focus is also on **Bt**-formulations and their active ingredients.

The US EPA reviewed toxicity/pathogenicity studies on **Bt** pesticides several times, most recently in 1998. Current data requirement for registration of (non-GM) microbial pesticides are included in the Federal Register (CFR) Title 40, Part 158. Information required by the EPA for registration do not involve the specific isolation of protein components for evaluation of toxic effects. The potential toxicity of proteins in the growth or fermentation medium can be evaluated by including the growth/fermentation materials in the dosing material for the acute oral, pulmonary, or intraperitoneal studies.

The data requirements are structured in a tiered testing system. Studies that are usually required in Tier I include acute toxicity tests (oral, pulmonary and iv injection (or intraperitoneal for larger microorganisms)) (McCLINTOCK 1998). Test animals are

investigated for mortality, body weight gain, clinical signs of toxicity (gross necropsy) and for the pattern of clearance of the microorganism from the animals. According to the US EPA the information from these acute toxicity studies allows an assessment for the potential of the microorganism to be pathogenic, or toxic, to mammals and from the lack of adverse effects the protein component of the microorganism are concluded to be non-toxic to mammals.

The FIFRA scientific advisory panel argued that there is no need for repeated dose testing if the results of the acute test are negative and no structural relationship to protein toxins can be identified (FIFRA 2000).

Considering the Cry toxins of **Bt** they act by solubilising in the insect mid-gut, proteolytic processing of a protoxin by mid-gut proteases, binding of the Cry toxin to mid-gut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores. Acute testing on rodents of Cry proteins produced from various GM and non-GM microbial sources failed to show adverse effects at high doses ranging from 3760 to 5220 mg/kg body weight via an oral route of exposure. Possible explanations for the low or lacking mammalian toxicity are, that activation of the protoxin requires an alkaline environment typical for insect gut but not for mammals; that for instance Cry1Ab protein is rapidly degraded in in-vitro digestibility studies, and that mammals are lacking the specific receptors in the gut necessary for toxic action.

As a general mode of action of toxic proteins, it is assumed, that proteins are susceptible to acid and enzymatic digestion to amino acids prior to assimilation. If toxicity testing of a protein is considered necessary, the acute exposure studies in laboratory animals are considered to be sufficient, since – if toxic – proteins are claimed to act via acute mechanisms only. This theoretical consideration obviously influence risk assessment practice. Yet it does not seem that this issue is thoroughly investigated (SUDAKIN 2003).

4 Key Aspects of Protein Toxicity Assessment

Over the last 10 years the overall approach and methods used in protein toxicity assessment in the context of GM plant and food has not been changing. Protein toxicity assessment in the EU is largely based on a set of indirect evidence (subsequently designated as ex-vivo studies), the US EPA and the FSANZ are asking for acute oral toxicity studies in rodents on top of this.

This approach presupposes that indications for toxic properties of a novel protein or for large uncertainties would be identified in the ex-vivo studies and alert assessors to conduct further tests. Such indications could include, for instance, unusual stability in IVD studies or to high temperatures, homology to known toxins, mode of action/specificity relevant for the human physiology, high-level exposure of humans, source organism of the protein known to be toxic or pathogenic, no prior history of human exposure. For the USA and Australia indications for toxicity could also be identified in the acute toxicity studies.

In more than 40 cases of protein toxicity assessment in the EU and the USA, no indications of toxic properties have apparently been identified which would have triggered further toxicity studies. Thus, there have been no test cases to challenge the robustness of this approach. From an in-depth analysis described in this report (see Chapter 2), however, certain weaknesses associated with the evidential basis used in toxicity assessment become apparent. These weaknesses are partly related to the methods applied, partly related to the type of evidence considered.

The US and FSANZ approaches, furthermore, presuppose that proteins – if toxic at all – are acting in an acute way only. A review of the database on protein toxicity in the scientific literature and in regulatory toxicology (in Chapters 1 and 3), however, does not suggest that this presumption is based on a body of systematic research.

This Chapter identifies and discusses key uncertainties and assumptions associated with protein toxicity assessment (see Sections 4.3 to 4.2). Section 4.3 explores possible parallels in protein toxicity assessment of GM plants and derived food, food enzymes and biopesticides.

4.1 Inherent uncertainties

Uncertainties in ex-vivo studies

So far there is little or no experience in the regulatory context with novel proteins

- From source organisms known to be pathogenic or toxic to human or higher animals.
- With no history of human consumption and/or no prior exposure to humans.
- With significantly modified amino acid sequences.
- With significant stability in IVD studies and/or studies on temperature stability.
- With homology to known toxins /anti-nutrients.
- With higher levels of human exposure.

A review of the bits of assumptions, methods and bits of evidence used in protein toxicity assessment revealed a number of weaknesses which are discussed at great length in Chapter 2 and will therefore only briefly mentioned here:

- In the absence of more detailed guidance the presently used method of IVD can lead to arbitrary results. Even more importantly, IVD studies are not validated and their results appear to be of little or at least questionable relevance for the human in-vivo context.
- Evidence from homology studies are quickly outdated but are still referred to after 10 and more years. Regular updates are perhaps conducted in industry on a voluntary basis but neither required nor provided to the authorities in case of renewals of applications or in case the same gene is expressed in other events or species. Furthermore, quality assurance and guidance in the conduct of such studies is lacking. Homology searches to anti-nutrients are not routinely conducted.
- Information on the mode of action of novel proteins and its relevance to the human body is illuminating but as the example of Cry1Ab show such studies can take a long time and are sometimes posing even more questions.
- The history of safe exposure/consumption often refers to anecdotal rather than scientific evidence. Thereby long-term and more mild toxic or adverse health effects would not necessarily become apparent. Furthermore, the protein with a particular history of consumption/exposure is not necessarily identical to the test protein. Here, the unresolved question what degree of homology and similarity in functional properties would be acceptable to cover a protein by such a safety claim.

Uncertainties associated with acute toxicity testing

According to experience in toxicology effects identified in repeated dose studies may not be detected in acute toxicity tests (SPÖK et al. 2003). This is also acknowledge by EU GMP risk assessors (e. g. see the SSC 2003 quote on p. 65f) who explicitly disregarded acute tests (EFSA 2006a).

Uncertainties associated with surrogate test proteins

Test proteins are routinely produced from microbes for reasons of feasibility and costs. This approach generates additional uncertainties as small changes in the primary sequence and in post-translational processing might not be detected. Furthermore, the set of methods used to prove protein equivalence differs between individual cases. This consequently translates into additional uncertainties in interpreting the results of the animal tests conducted using this type of test protein.

As large scale protein purification techniques advances and with GM plants expressing novel proteins at higher levels it might become possible to purify the novel proteins from plants instead of microbes.

4.2 Underlying assumptions

Protein toxicity assessment in the context of GM plants and food is based on some explicit and implicit assumptions which deserve to be explored.

4.2.1 General presumption of no-harm

Novel proteins are so far “not generally associated with safety concerns when consumed orally” mainly because of the high protein content in human diet (KÖNIG et al. 2004). Still, it is recognised that there are proteins associated with toxic, anti-nutrient, pharmacological and allergenic effects. Therefore, the potential for adverse effects should be investigated (ibid.). This assumption has been translated into toxicity assessment which heavily relies on indirect evidence.

This is contrasted by approaches in other regulatory contexts such as chemicals. Here, novel substances are either deemed dangerous unless proven safe or considered to be of unknown toxic properties which will then be identified in hazard identification by a more rigorous set of animal tests.

Given the fact that risk assessment documents do not deny that there are proteins which can be harmful to human (beyond allergenic effects) this means that there is confidence that the data required to characterise the protein (characterisation of the source organism, history of human consumption or exposure, mode of action of the protein, homology comparison to a database of known toxins, and in-vitro digestibility studies) will alert risk assessors and trigger more extensive or additional (animal) toxicity studies. Whether this approach would actually work in practice is difficult to say as there seems to exist not a single case of sufficient concern. Thus, acute toxicity studies have been accepted so far. Some of the US applications were even approved without any animal toxicity tests.

In any case it puts emphasis on the reliability, quality and relevance of the data for characterising the novel protein. In the light of the critical review of some of the methods used to generate these data (as discussed in Chapter 2) there seems to be at least some leeway for improvements.

4.2.2 Proteins are acting as acute toxicants (only)

Another assumption occasionally made explicit is that toxic proteins do have acute effects. Guidance documents and risk assessment dossiers are frequently referring to SJOBLAD et al. (1992) as if there exists not other than acute toxic of proteins at all (e.g. BRENT et al. 2003, HÉROUET et al. 2005). In fact the original quote states that

"if toxicity testing of a protein is considered necessary then acute exposure studies in laboratory animals should be sufficient, since – if toxic – proteins are known to act via acute mechanisms" (p. 8).

However, this does, not mean that other than acute toxic effects of proteins are excluded.

Assuming the statement correctly reflected the state of scientific knowledge at the time⁴³ it only means that there is no evidence of other than acute mechanisms. It does not imply that there has been any research nor does it comment on the quality of the knowledge base backing this statement. It could simply mean that in fact there have been no reports in the scientific literature. Acute and massive toxicity of proteins might easily be detected without any investigations, more subtle effects only showing up after repeated ingestion would not be detected without epidemiological and other scientific studies.

In a very similar way PARIZA & JOHNSON (2001) justified their recommendation of acute oral toxicity tests in their review on toxicity assessment of food enzymes as follows:

"It should be emphasized that this test is not intended to establish a LD50. Rather it is a specially designed test for determining safety of bacterial enzymes, since with rare exception the only toxins known to be produced by bacteria are proteins or peptides (enterotoxins and certain neurotoxins) which are acute toxins that are produced by only a few bacterial species" (PARIZA & JOHNSON 2001: 181).

⁴³ This statement is probably wrong as it does not consider allergic sensitizing properties of proteins which are not acute effects, a prerequisite for allergic effects, which are also considered in toxicology. However, in GM plant and food risk assessment toxic and allergic properties are considered separately. Furthermore, immunogenic effects of proteins are explicitly not included in the scope of this report.

Here, only bacterial proteins are considered and the existence of non-acute toxins is acknowledged.

A related aspect is the calculation of NOELs based on acute toxicity instead of repeated dose studies (see Section 2.11).

Given its important role the Sjoblad claim is worth to be investigated further. Some essential questions can be asked:

- Is there any evidence contradicting the claim of SJOBLAD et al. (1992)?
- What kind of sub-acute and long-term toxic effects of proteins are described in the literature?

These questions are briefly discussed in the following.

Evidence from scientific publications

Toxic proteins effective via the oral route have usually been described in the scientific literature in case of clinically relevant symptoms following ingestion of contaminated food or water. Protein toxins from a variety of bacteria have been investigated because they are important virulence factors. Some toxins have also been studied for various medical applications.

Taking the quote of SJOBLAD et al. (1992) literally there are definitely proteins which are exerting toxic effects beyond acute toxicity, prions and proteins exerting immunogenic effects. Prions are however considered to be a special group of proteins which are not deemed to be relevant to this report and immunogenic effects of protein are explicitly excluded from the scope of the report.

As described in Chapter 1 many known toxins of plant and microbial origin do exert acute toxic effects with some of them also exerting toxic effects beyond acute toxicity. Nevertheless, from published scientific papers there does not seem to exist a strong evidential basis on proteins exerting toxic effects beyond acute toxicity. This conclusion does however not appear to be founded on strong scientific grounds, as there does not seem to be significant research into long term effects of toxic proteins.

Another particularly interesting case are lectins. Some lectins are clearly causing toxic effects, such as ricin and abrin. Other lectins and also some proteinase inhibitors cause adverse health effects which might be mild but nevertheless relevant, e.g. growth of pancreas and gastrointestinal disorders. Consequently, lectins are frequently considered to be either toxins or antinutrients though it might be difficult to draw a sharp line between toxic and anti-nutritional effects. As anti-nutritional properties of the whole GM plant are an issue in risk assessment some applicants have extended the scope of sequence homology comparisons to include not only known toxins but also known anti-nutrients.

Evidence from regulatory science

Toxicity tests conducted on proteins in the course of pre-market risk assessments of food enzymes, biopesticides (see Chapter 3) and GM plants seem to suggest that proteins used in food contexts or as biopesticides do not exert any toxic effects at all. This conclusion might however be premature.

First, the cases reviewed in this report suggests a skewed picture as it cannot be expected that in case of toxic effects for humans (at least in case of food enzymes and GM plants) product development is not likely to continue. In case of food enzymes it is furthermore unlikely that any adverse effects identified in JECFA reviews would be reported by JECFA or in the scientific literature (FAO, pers. communication). Moreover,

it is quite likely that enzymes from known or suspected toxicogenic microbes are less likely to be developed for commercial food production purposes. The same is true for GM plants.

Second, in case of novel proteins introduced into GM plants only a very small number of proteins have been subjected to a toxicity assessment in the course of pre-market authorisation, so far. Not more than 40 proteins, most of them having some kind of history of human consumption, have been assessed. Given the large variety of proteins which will be potentially interested for market commercialisation (e.g. indicated by more than 1400 different genes introduced in plants and registered in the course of US field trials) this might not be representative in any way.

In case of food enzymes, the large number of food enzymes that were so far subjected to toxicity testing represent a small range of enzyme classes. Food enzymes presently used comprise only a tiny fraction of total enzymes identified so far; about 60 of a total of 4800 catalytic different types listed by enzyme databases (<http://www.brenda-enzymes.info/>). About two third of all food enzymes presently in use are hydrolases and belong to only one of six main enzyme classes according to IUBMB classification. At least 247 different food enzymes are presently on the market compared to more than 12000 entries in an enzyme database (see also SPÖK & PROKSCH 2007).

In both contexts an increasing number of proteins can be expected which do not have a history of human consumption. In case of GM plant this is definitely the case with GM plants intended for certain industrial applications, e.g. maize 3277 for using maize as an energy crop and in case of production of pharmaceutical and other industrial proteins by GM plants (plant molecular farming). In case of food enzymes more and more enzymes are selected from extremophiles.

Given the discussion above it can be concluded that there is little evidence of toxic effects of proteins beyond acute toxicity and following exposure via the oral route. However, given limited knowledge database, the lack of specific research and the perhaps overestimated relevance of safety reassurance from regulatory toxicology is perhaps premature to agree to the claim of SJOBLAD et al. Moreover there are proteins which are likely to exert adverse effects some of which might not be considered to be covered in toxicity assessment.

4.3 Toxicity assessment of novel proteins in context

Chapter 3 in this report describes toxicity assessment of novel proteins in other regulatory contexts. This Section explores similarities and differences between these approaches and discusses to what extent the assessment approaches in food enzymes and biopesticides might be a useful comparator for novel proteins in GM plants and food.

In case of food enzymes assessment essentially includes a kind of standardised package of toxicity tests, usually a 90-day oral toxicity study on the basis of which a NOEL is calculated and two in-vitro genotoxicity tests (see Chapter 3.1). Based on worst-case calculations for human exposure a minimal margin of safety can then be calculated. Indirect evidence such as in-vitro digestibility studies and homology comparisons are not required.

In case of biopesticides (US EPA) a tiered approach is being followed with Tier 1 requirements being essentially similar to what is presently proposed for novel proteins in GM plants and food. The main differences are due to the fact that in case of products made of cells or spores a pathogenic potential is also considered. This is however, not

entirely surprising as the US EPA developed its requirement for PIPs alongside and on the basis of requirements for microbial pesticides.

In the context of GM plants and food novel proteins are assessed in a case-by-case and weight of evidence approach allowing for individual assessments and providing a high level of flexibility to applicants and risks assessors what kind of data will be provided. In practice however, fairly the same data packages have been provided in the EU and the USA as well as over time. Exceptions are essentially limited to acute toxicity studies which are required by the US EPA for **Bt** proteins only.

As with microbial biopesticides in the USA animal testing is set to a minimum or even not necessary if the body of evidence does not indicate any concern. This essentially include characterisation of the source organism, history of human consumption or exposure, mode of action of the protein, and homology searches to a database of known toxins, and in-vitro digestibility studies. In the absence of relevant concern, so far single-dose acute toxicity studies in rodents were deemed acceptable in the USA. In the EU acute studies are included in the dossiers but neither required by guidance documents nor by EFSA.

The main contrast here appears to be the reliance on indirect evidence in case of GM plants and food and the emphasis on animal studies in case of food enzymes with the former approach allowing for a more sparingly application of animal studies and the latter suggestion a higher level of safety. Furthermore, repeated dose tests appear to be more relevant for assessing food compared to a single-dose tests and thereby more realistically mirrors real world exposure.

But, are novel proteins from GMP and food and food enzymes in fact comparable? The answer would perhaps be “yes, partly”. Most food enzymes are used as processing aids, thus present as residues in the final food only. If nevertheless assuming that all food enzymes used will be present in the final food stuff (as routinely assumed for estimates of the EDI and calculation of the Margin of Safety) daily intake yield similar numbers compared to analogue calculations for novel plant proteins and derived food (see Table 24). Similarly, the concentration of the food enzyme in food is in a similar range compared to the concentration of novel proteins in GM plants, which is of particular relevance for plants being consumed raw (e.g. sweet maize, tomatoes etc.).

Table 24: Worst case estimates of heterologous proteins in food stuff.

Food enzymes			
TOS (mg / kg) in food	Active protein (mg / kg) in food	EDI (mg TOS / kg bw / day)	Application
0.02–2000 ^a	0.00005–0.15 ^a	n.a.	General range
5.7	N.sp.	0.015	In bread
102	N.sp.	0.27	In bread
400	N.sp.	0.318	In syrup
25	N.sp.	0.318	Pullulanase in beer
0.0188	N.sp.	0.005	Lipase in bread
Novel proteins in GM plants			
	Active protein (mg / kg) in edible plant tissue	TMDI (mg / kg bw) (b)	
N.a.	0.01–37	0.0000024–0.0089	General range
N.a.	828–1627	200–390 (c)	Amylase in maize 3272 kernels

N.a.: not applicable; n.sp.: not specified; TMDI: Theoretical Maximum Daily Intake; TOS: total organic substance; a) calculated on the basis of general ranges provided in FEDERAL ENVIRONMENT AGENCY/IFZ (2002) and in AMFEP (2006b); b) calculated based on EU TMDI; c) this event is not intended for food; numbers are calculated based on a worst case scenario of accidental commingling with food maize and that the maize TMDI of a given person will only consist of products containing this maize variety.

Source: based on SPÓK & PROKSCH (2007), extended and modified.

Thus, in terms of exposure there are apparent similarities. Given its predominant application as processing aids, food enzymes might be even less likely to be present as active proteins in the final food compared to novel proteins in GM plants, especially if there is little or no processing.

In case of food enzymes the toxicity testing package is, however, not only addressing the enzyme protein itself. Possible contaminations of the enzyme isolate (the purified enzyme from the fermentation broth), especially bacterial toxins and mycotoxins are the in fact of greater concerns (RASMUSSEN & SKOVGAARD 1996, PARIZA & JOHNSON 2000). For enzymes of very high purity and specificity the full toxicity testing may not be needed (SCF 1991, RASMUSSEN & SKOVGAARD 1996). In case the production microorganism has a long history of safety in food use, belongs to a species where no toxins are produced, and is of well documented origin waivers from toxicological testing are possible (SCF 1992, EFSA 2006b).

PARIZA & JOHNSON (2001) suggest conducting acute toxicity tests instead in case of bacterial enzyme preparations as “with rare exceptions” bacterial toxins (mostly proteins) are known to be acute toxic. Furthermore, the authors suggest giving up genotoxicity testing based on a review of more than hundred tests conducted on enzymes.

Despite such suggestions to reduce toxicity testing, the endpoints investigated did not change in practice, still 90-day subchronic tests and two tests for genotoxicity are routinely conducted.

Thus, the different rationales in toxicity testing might have reason beyond the proteins.

More interesting in this respect are the differences between toxicity assessment for food enzymes and the requirements laid out in the recent EFSA guidance on food/feed products derived from GMM. Despite the apparent overlap in the products that might be considered under both guidance documents, the rationale of the EFSA guidance is very similar to its guidance on GM plants and derived food, proposing essentially the same data and testing requirements for toxicity assessment of novel proteins in GMM and

GMPs in both documents. However, this divergence is of less relevance to this report and will therefore not be discussed here.

Another interesting difference is the way risk characterisation is conducted. In case of food enzymes risk characterisation is conducted according to normal toxicological practice and based on a NOAEL derived from 90-day subchronic toxicity studies and an estimate of expected exposure from which then the MoS is derived. In the context of GM plants and food the main difference is the absence of such a formal approach in earlier dossiers and the less extensive estimates of dietary intake. In more recent dossiers a NOAEL is derived based on acute toxicity tests (and sometimes another NOAEL is derived from a 90-day subchronic study of the whole plant). Here the key issue is the relevance of a NOAEL derived from acute toxicity testing to exposure from food or food components.

5 Conclusions and Recommendations

Protein toxicity assessment in the context of GMOs is operating on two main assumptions: (i) orally ingested proteins are not generally associated with safety concerns and (ii) if proteins are toxic at all they are acting by acute mechanisms only. However, the evidential basis supporting these assumptions seems to be rather weak. In the context of regulatory toxicology experience is virtually limited to a small range of proteins comprising biopesticides and food enzymes (see Chapter 4). In case of GM crops experience is so far limited to some 40 proteins. Toxic properties of proteins via the oral route and beyond acute toxicity have not been a target of systematic scientific investigations. Evidence possibly contradicting these assumptions comes e. g. from lectins.

In the absence of a significant number of test cases such as proteins with little or no history of human exposure and from other than bacterial sources, it has to be established that the presently applied approach in toxicity assessment would be able to detect non-acute adverse effects and to guard against surprises.

These uncertainties are not reflected in the presently applied toxicity assessment which heavily relies on indirect evidence and ex-vivo studies. The most meaningful studies for toxicity assessment, in-vivo studies, are either not compulsory or are aiming at acute toxicity only. Whether in-vivo studies would be required depend on the results of the ex-vivo (and the acute tests). A detailed analysis of the toxicity assessment approach revealed weaknesses (for a comprehensive list and description see Chapter 3), in particular:

- Results of homology searches are quickly outdated and interpretation of results is not clear
- Test proteins from bacterial sources are not fully representative of the plant-derived protein
- Variable practice and questionable relevance of in-vitro digestibility tests
- Exposure assessments and risk characterisation does not correspond to normal toxicological practice

The analysis also identified a lack of guidance, in particular for homology comparisons and in-vitro digestibility tests.

These limitations in protein toxicity assessment call for and should guide further improvements of the assessment approach, the methods applied and the testing practice. This is of particular importance with increasing diversity and “novelty” of proteins in the pipeline. Improvement can be achieved by conducting further research, developing and fine-tuning of methods and establishing guidance.

As long as the protein toxicity assessment approach has neither been tested nor improved it might be considered to conduct repeated-dose tests according to OECD protocols (with the duration depending on the evidence available) as a standard requirement for assessing novel proteins. The methods available to purify plant proteins and the costs should be more thoroughly reviewed and compared to microbial derived proteins. If expression of the plant protein is sufficiently high (depending on the progress in plant-protein purification methods from plant molecular farming even for lower expression rates) test proteins should be produced from the GM plants.

Further research would particularly be needed in order to:

- clarify more properly any toxic effects of proteins including those beyond acute effects.

- clarify the relevance of IVD for the in-vivo situation in humans and possible alternatives.
- clarify the fate in and absorption of proteins from the GI tract of humans.

More detailed guidance would be needed on:

- Presently applied IVD studies (laboratory protocol, chemicals used, validation).
- Homology searches (databases, parameters, verification, quality assurance, updates).
- On the robustness of evidence to which one could legitimately refer if relying on the history of consumption/exposure; and on the acceptability of distant relationships between the protein with a history of exposure and the test protein.

Clarification would be needed:

- To differentiate more clearly between toxic and anti-nutritive properties of proteins.
- On the explicit need and methods to assess possible anti-nutritive properties alongside toxic properties of novel proteins.

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Part B: Assessment of ecotoxicity

7 Protein Ecotoxicity – Ecotoxic proteins

7.1 Plant proteins

7.1.1 Types of plant defences

The structural defence of plants can be distinguished from chemical defence mechanisms. Resistance to insect pests is achieved by modifying the behaviour of the animal on the host plant or affecting the quality of the plant as a resource. Thus plant components aim at repelling or deterring insect pests or, if this fails, the degree of the damage is limited by antifeedants making the plant a poor source for the insect. Plant proteins play key roles both as constitutive factors and as active response mechanisms to pest and pathogen attack.

Mechanisms for protection against insects involve secondary compounds such as alkaloids, isoprenoids, non-protein amino acids but also proteins (GATEHOUSE et al. 1990). Within proteins accumulated in plant seeds, protease inhibitors, lectins, lectin-like proteins and ribosome-inactivating proteins (RIPs) have been shown to possess antimetabolic or toxic effects against insects (Sousa et al. 1993). Other proteins involved in defense mechanisms of plants are the arcelins, chitinases, canatoxin and modified forms of storage proteins (CARLINI & GROSSI-DE-SÁ 2002). Some of these insect-protective proteins have been lost during the domestication process, e.g. by silencing of the respective encoding genes, but can be reactivated if the expression of other proteins is changed (CARLINI & GROSSI-DE-SÁ 2002).

A frequent distinction in plant defence against other organisms is the constitutive and the inducible defence system although there is a considerable overlap between them.

The constitutive protective system can be described as organ-, tissue- or cell type-specific and is not related to infection or damage. This type of plant defence is frequently found in plant seeds and storage tissues but also in vegetative tissues as well as in gums and latexes. These compounds are mostly inhibitors of hydrolytic enzymes which are classified into families according to their amino acid sequence and specificities and are mostly specific for proteinases such as trypsin, chymotrypsin, cysteine-proteinases or alpha-amylases. Some proteins are also bifunctional, inhibiting both, proteinases and amylases, or other enzymes such as chitinases.

The second defence mechanism is switched on only following a pest or pathogen attack thus exhibiting a more regulated expression pattern and a specific protective role. These proteins can be classified into several types, such as beta-1,3-glucanases, chitinases or antifungal proteins. Other proteins include the protease inhibitor II of potato, a trypsin and chymotrypsin inhibitor induced by mechanical leaf damage but also expressed in tubers and flowers. Another example are the pathogenesis-related proteins (PR-proteins) induced by microbial infection or by certain chemical elicitors such as acetyl salicylic acid. These proteins have a range of common features, such as low relative molecular mass, acidity, are extractable at low pH, show high resistance to proteolysis and are located in intercellular spaces although basic forms are also located in the vacuole.

7.1.2 Lectins

A number of lectins have been shown to kill or inhibit the growth of insect pests. Reviews of the insecticidal properties of lectins can be found in GATEHOUSE et al. (1995), CARLINI & GROSSI-DE-SÁ (2002) and VASCONCELOS & OLIVEIRA (2004).

In particular, the following lectins receive much attention in the literature with respect to their possible insecticidal activity (short name, source): wheat germ agglutinin (WGA, *Triticum vulgare*), snowdrop agglutinin (GNA, *Galanthus nivalis*), the pea lectin (PSA, *Pisum sativum*) and the bean lectin (PTA, *Phaseolus vulgaris*). Furthermore, lectins from *Ricinus communis*, from the Jackbean (Concanavalin A, Con A) but also other less prominent lectins have potent ecotoxic properties.

Type 2 Ribosome inactivating proteins (RIPs), a special type of lectins, are known to be extremely potent cytotoxins while type 1 RIPs are considered to be less potent toxins. Recently lectins with very low cytotoxicity were identified as type 2 RIPs (see review in STIRPE 2004). Prominent RIPs are ricin from castor bean seeds (*Ricinus communis*) or abrin from the Jequiriti bean (*Abrus precatorius*).

7.1.2.1 Occurrence and role

Lectins are particularly abundant in Leguminosae, although they have been also found in viruses, bacteria, invertebrates and vertebrates. Lectins from different plant sources show a considerable degree of structural similarity despite differences in carbohydrate-binding specificities (CARLINI & GROSSI-DE-SÁ 2002). Although lectins occur predominantly in ripe seeds and in other storage organs, they have been found also in leaves, roots and other organs of the plant. Specifically, ribosome inactivating proteins (RIPs), a special type of lectins, have been detected in Angiospermae, in mushrooms and in algae ranging from traces to hundreds of milligrams per 100 g and are present in specific, many or even all tissues examined (see review in STIRPE 2004).

Besides direct evidence of the plant defence function of lectins, there is indirect evidence of the protective role of lectins against pests and pathogens due to their occurrence in those parts of the plant which need extra protection due to their specific role for the survival of the individual and their lack of an active defence system (PEUMANS & VAN DAMME 1995). There is also evidence that lectins have relevance for physiological processes such as cell-to-cell recognition in pollen physiology, symbiotic interactions and receptor binding (see PUSZTAI 1991 cited in RAHBE et al. 1995). Another proposed role of some legume lectins is the specific interaction between legumes and their symbiotic nitrogen-fixing bacteria and possibly the function as storage proteins (WANG et al. 2003, PEUMANS & VAN DAMME 1995).

In maize kernel endosperms the inactive pro-enzyme of a ribosome inactivating protein (RIP) can be enzymatically activated. Activation by endogenous plant proteases occurs during germination when the storage protein reserves are most vulnerable to pests and pathogens. The activated enzyme is at least 100fold more active than the pro-RIP (DOWD et al. 1998). Maize endosperm RIP can be activated by papain, a cysteine protease. As cysteine proteases are not present in caterpillars but in several beetle species, this has been suggested as the possible reason for the activation of the pro-RIP in the gut of beetles (DOWD et al. 1998).

7.1.2.2 Classification

Plant lectins are high-molecular compounds. They are carbohydrate-binding proteins that bind reversibly and specifically to mono- or oligosaccharides. The classification of lectins is based on their carbohydrate recognizing and binding ability (PEUMANS & VAN DAMME 1995). Typically lectins are multivalent and are able to agglutinate cells (CARLINI & GROSSI-DE-SÁ 2002).

It has been suggested that the presence of at least one non-catalytic domain that binds reversibly to a specific carbohydrate is the only prerequisite for a protein to be named a lectin (PEUMANS & VAN DAMME 1995). Consequently three types of lectins can be distinguished:

- Merolectins: containing a single carbohydrate-binding domain.
- Hololectins: containing two or more very similar or homologous carbohydrate-binding domains.
- Chimerolectins: containing a carbohydrate-binding domain and an independently acting unrelated domain with catalytic activity.

A fourth type of lectins, the superlectins, has also been suggested which possess at least two carbohydrate-binding domains but their sites are able to recognise structurally unrelated sugars (VAN DAMME et al. 1998 cited in VASCONCELOS & OLIVEIRA 2004).

Furthermore, plant lectins are also divided into groups according to their affinity to monosaccharides, to which they bind on cell surfaces. Several distinct groups of structurally and evolutionary related proteins among the lectins have been described: legume lectins, chitin-binding lectins, type 2 RIPs, monocot mannose-binding lectins, jacalin-related lectins, amaranthin lectins and cucurbit phloem lectins.

7.1.3 Enzyme inhibitors

Proteins that inhibit hydrolytic enzymes, especially alpha-amylases and proteinases, are particularly widespread in seeds and other plant storage tissues but can also be found in vegetative tissues of plants. In plants proteinase inhibitors occur mainly in legumes and cereals. They have also been reported from animals and micro-organisms. Additionally, bifunctional inhibitors (alpha-amylase/proteinase inhibitors) are relatively common.

Plant enzyme inhibitors can be classified into families based on their amino acid sequences and their specificities. They act specifically on proteinases, mainly serine proteinases such as trypsin or chymotrypsin, but can also inhibit other enzymes such as carboxypeptidases, cysteine proteinases or alpha-amylases.

7.1.3.1 Alpha-Amylase Inhibitors (alpha-AIs)

Occurrence and role

Alpha-amylases are enzymes that catalyse the hydrolysis of alpha-D-(1-4)-glucan linkages in starch components, glycogen and other carbohydrates. Therefore these enzymes play a major role for the carbohydrate metabolism of animals, especially insects that feed on starchy plant seeds. In plants such inhibitors are mainly present in cereals such as wheat, barley, rye and rice but also in Leguminosae such as peas and beans (FRANCO et al. 2002). They are responsible for the protection of seeds against exogenous proteinases and alpha-amylases produced by various pathogens and pests.

Classification

Alpha-amylases are classified by their tertiary structure into six classes: lectin-like, knottin-like, cereal-type, Kunitz-like, γ -purothionin-like and thaumatin-like inhibitors (see FRANCO et al. 2002).

7.1.3.2 Proteinase Inhibitors (PIs)

Occurrence and role

Proteinase inhibitors are proteins or groups of proteins also occurring mainly in legumes and cereals. They occur in high quantities mainly in reproductive organs but can be induced also in leaves by infection or damage. PIs are considered essential components of the plant defence against insects, protecting seeds during dormancy or responding to wounding of the plant upon infection or damage. PIs are made responsible for resistance against certain insects and pathogens or for protection against digestion when eaten by birds and other herbivores. They are supposed to confer protection against proteolytic enzymes of parasites and insects but are also involved in the

regulation of endogenous proteinases during seed dormancy. Other proposed roles include their function as storage or reserve proteins, reserve protein mobilization and regulators of proteolytic activity and developmental processes. Involvement in regulation of programmed cell death, such as protecting the plant from cell death as induced by pathogen infestations or by oxidative stress has also been suggested (HAQ et al. 2004). Proteinase inhibitors are mostly thermo-labile, but some have a high stability against heat, acids or bases.

PIs can be expressed constitutively or tissue-specifically. Highest levels are usually found in very young leaves. Although their expression is often not related to infection or damage, some proteinase inhibitors are induced by mechanical damage of leaves. They are also present in tubers and flowers. Consequently, a clear division into constitutive and tissue-specific and inducible proteins is not possible, because proteins are often expressed during development of storage tissues while being inducible in other tissue types (SHEWRY & LUCAS 1997). For example, transcripts of tomato protein inhibitors are not detectable in unwounded leaves but accumulate at leaf injury sites with maximum levels at eight hours after damage whereas in systematically responding tissue the translation products show a rather transient abundance in unwounded leaves (BOWLES 1990).

Classification

The classification of PIs into four classes is based on the specificity for each of the proteolytic enzyme and on the active amino acid in the “reaction center”. PIs are classified into serine proteinases inhibitors (serine, histidine), cysteine proteinases inhibitors (cysteine), aspartic proteinases inhibitors (aspartate group) and metallo-proteinases inhibitors (metallic ion). The activity of the PI is due to the formation of stable complexes with the target proteases.

Serine proteinases inhibitors are the most common and most-studied class of PIs. Serine PIs can be further classified into different families based on their amino acid sequences such as the Bowman-Birk family, the Kunitz-family, Potato I and II families, curcubit family, cereal family and others (SHEWRY & LUCAS 1997). The structure of the reaction center and the mechanism of action are well preserved among serine PIs (CARLINI & GROSSI-DE-SÁ 2002).

Cysteine proteinase inhibitors, also named plant cystatins or phytocystatins, are the second most-studied class of PIs. Their occurrence has also been described from crops plants, such as sunflower, rice, wheat, maize and soybean.

In contrast to serine and cysteine PIs, aspartic PIs are relatively rare in plants. It is known that potato tubers possess cathepsin D, an aspartic PI. Also metallo-proteinase inhibitors are relatively rare and have been described from tomato and potato plants (HAQ et al. 2004).

Bowman Birk type inhibitors are small polypeptides (around 10 kDa) characterised by seven disulphide bonds and two reactive sites that are specific for different proteinases (trypsin, chymotrypsin). They were used for first attempts to introduce resistance to insects using GM plants (HILDER et al. 1987). In transgenic tobacco plants the cowpea trypsin inhibitor inhibited the growth of tobacco budworm larvae (*Heliothis virescens*) and to some extent also of another lepidopteran species *Helicoverpa zea*.

Kunitz-type inhibitors are larger proteins (around 20 kDa) and are present in a range of legume species, mostly in soybean. They are made of one or two disulphide-linked polypeptide chains and possess a single reactive site for trypsin but chymotrypsin is also weakly inhibited.

7.2 Fungal proteins

Fungi excrete a wide range of toxic metabolites and are used in insect control. Examples include *Beauveria bassiana* for the control of numerous insects of Coleoptera, Lepidoptera and Diptera or *Metarhizium anisopliae* for the control of rice pests or the Colorado potato beetle.

Several fungal proteins or peptides have been shown to exhibit insecticidal properties. Important proteins with known ecotoxic effectiveness are Hirsutellin A (see references in LIU et al. 1996, MAZET & VEY 1995), Bassiacridin (QUESADA-MORAGA & VEY 2004), Beauvericin (see references in GANASSI et al. 2002) or the destruxins.

Bassiacridin and Hirsutellin A are composed of single polypeptide chains with different molecular masses. While Bassiacridin shows no protease or peptidase activity, three enzyme activities are reported for Hirsutellin A. Hirsutellin A is basically completely stable and shows unchanged activity either when incubated at high or low temperatures and resists treatment with different enzymes (MAZET & VEY 1995). In contrast Bassiacridin seems to be susceptible to heat treatment (QUESADA-MORAGA & VEY 2004).

7.3 Cyanobacterial and bacterial proteins

7.3.1 Cyanobacterial proteins

Several toxins have been described from Cyanobacteria. They are mainly contained in the cyanobacteria *Microcystis*, *Oscillatoria*, *Nodularia* and *Anabaena* (Codd et al. 2005). Cyclic hepta- and pentapeptides can be distinguished. The hepta-peptides are commonly termed as Microcystin, possessing a common structure that contains D-amino acids and the alpha-amino acid *Adda*. Also Nodularin has been described from the genus *Nodularia* as a toxic protein. Microcystin consists of seven amino acids and more than 70 structural variants have been described, while the structurally closely related Nodularin shows less structural variation (WIEGAND & PFLUGMACHER 2005).

7.3.2 Bacterial proteins

Many bacterial species produce insecticidal toxins. Although many species of entomopathogenic bacteria have been described, bacteria used in microbial insecticides are largely restricted to the genus *Bacillus*. Three main species are used: *Bacillus popilliae*, *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus*. Recently, a new class of proteins conferring insecticidal activity has been described from *Photorhabdus* spp., *Xenorhabdus* spp. and *Serratia entomophila* (FFRENCH-CONSTANT et al. 2007).

Photorhabdus and *Xenorhabdus* bacteria are symbionts of entomopathogenic nematodes and release toxins into an insect upon invasion by the nematode. Inside the insect the bacteria are released from the infective nematode, replicate and release a range of toxins to kill the insect host. After some generational cycles the nematodes take up the bacteria again and search for new hosts. The potential use of these toxins for crop protection in agriculture by the expression in transgenic plants as an alternative to *Bt*-toxins has been suggested (FFRENCH-CONSTANT et al. 2007).

7.3.2.1 Endotoxins of *Bacillus thuringiensis*

Occurrence and role

Bacillus thuringiensis (*Bt*) are gram-positive bacteria with entomopathogenic properties. They are found in a wide variety of soils, on plant surfaces and in insect cadavers.

Bacillus thuringiensis strains contain a range of insecticidal toxins. The presence of crystal or non-crystal proteins and the spores contributes to the insecticidal activity of

Bt strains (DULMAGE 1981). In some cases the spores act in synergy with the endotoxins (KOZIEL et al. 1993).

Bacillus thuringiensis undergoes a vegetative and a stationary phase. During the sporulation phase **Bt** produces parasporal crystals. These crystals are typically comprised of one or more delta-endotoxins, the Crystal (Cry) toxins and Cytolytic (Cyt) toxins. These proteins are encoded by **cry** or **cyt** genes located mainly on plasmids and are generally expressed during the stationary phase. The gene products accumulate in the mother cell to form a crystal inclusion that can account for 20–30 % of the dry weight of the cells. The delta-endotoxins are released with the spores when the bacterial cells ultimately lyse. The overall insecticidal activity of a **Bt** strain is considered due to the additive and/or synergistic interactions of the individual delta-endotoxins (KOZIEL et al. 1993) but these endotoxins are considered only one of several insecticidal proteins produced by the bacteria allowing development in dead or weakened insect larvae (SCHNEPF et al. 1998).

Classification of **Bt** toxins

The original classification system of **Bt** genes by HÖFTE & WHITELEY (1989) was based on the toxin gene sequences related to the activity against susceptible insects. Four major classes based on their insect specificity were established: **cryI** (Lepidoptera), **cryII** (Lepidoptera and Diptera), **cryIII** (Coleoptera) and **cryIV** (Diptera). Later two new classes were added (**cryV** and **cryVI**). This system was replaced by a systematic nomenclature only based on the amino acid sequence similarity of the gene products (CRICKMORE et al. 1998). Ranking is based on the degree of homology which needs to be shared to have the same Arabic number, uppercase letter or lowercase letter without parentheses (e.g. cry1Ab). A quaternary ranking has been introduced for toxins with identical sequences but isolated separately (e.g. cry1Ac2). Also binary toxins are included in the classification system, if together they exhibit a significant toxic effect (e.g. Cry34Ab/Cry35Ab)

About major 50 classes for **cry** genes, **cry1** through **cry49** and two major classes of **cyt** genes, **cyt1** and **cyt2** are designated by this classification system. More than 300 different **cry** genes and more than 20 **cyt** genes have been described (CRICKMORE et al. 2007).

Insecticidal toxicity of separate **Bt** strains differs considerably as different isolates of the same **Bt** variety can produce different endotoxins (DULMAGE 1981) and the same toxins can be found in different strains or subspecies (KOZIEL 1993). The parasporal crystal inclusions usually contain more than one delta-endotoxin and the corresponding delta-endotoxin genes are usually carried on plasmids which can be exchanged among different strains. Consequently, subspecies or serovars classification (e.g. **kurstaki**, **israelensis**, etc.) does not allow predictions of the **cry**-gene content.

Two size classes can be distinguished, toxins with a size of 130–140 kDa and toxins with a size of approximately 70 kDa. While the N-terminal half of the protein is conserved, the C-terminal half is a domain that is not found in the smaller toxins (PIGOTT & ELLAR 2007). Most of the Cry endotoxins share a similar structure with three domains. The three domains are involved in different roles in the mode of action against a specific insect but their exact role and functioning is still under discussion.

8 Ecotoxicity modes of proteins

8.1 Plant proteins

8.1.1 Lectins

The typical characteristic of lectins is their specific carbohydrate-binding activity. The binding of the lectin to a specific receptor either within or outside the plant plays a key role in the interaction with other organisms (PEUMANS & VAN DAMME 1995). The receptors are defined as glyco-conjugates that possess a carbohydrate moiety with a complementary structure to that of the lectin binding site (PEUMANS & VAN DAMME 1995). Lectins are therefore the only plant proteins (except some enzymes) that can recognize and bind receptors on the surface of micro-organisms or on the intestinal tracts of herbivores. Thus many lectins play a role in plant defence and have antimicrobial activity or confer resistance to insect pests (SHEWRY & LUCAS 1997). This is also supported by the high affinity of many lectins for oligosaccharides which are absent in plants but common or typical in other organisms, such as chitin, a major component of the cell wall of fungi or the skeleton of invertebrates (PEUMANS & VAN DAMME 1995).

Plant lectins can possess antiviral, anti-bacterial, anti-fungal or insecticidal activity. The anti-viral activity is possibly due to glyco-proteins in the virions of animal and human viruses. The anti-bacterial activity has been suggested through interactions with cell wall carbohydrates or other bacterial cell wall components such as peptidoglycans or by blocking of bacterial motility. The anti-fungal activity, especially of chitin-binding plant lectins such as the nettle lectin (*Urtica dioica*), has been demonstrated *in vitro*, although the exact mechanism is unknown (PEUMANS & VAN DAMME 1995).

Insecticidal properties of plant lectins have been described by many authors. Effectiveness of plant lectins has been shown for several insect orders. Three possible mechanisms of actions of lectins with respect to their insecticidal activity have been suggested so far:

- Binding of lectins to the chitin of the peritrophic membrane (for chitin-binding lectins only) or binding to glyco-conjugates on the epithelial cells in the digestive tracts (brush border membrane).

Binding of the lectin to the midgut epithelial cells or binding to the peritrophic membrane in the midgut region have been suggested as possible mechanisms (CZAPLA & LANG 1990, GATEHOUSE et al. 1995). Lectins administered to Dipteran larvae were shown to bind to the peritrophic membrane and to reduce its permeability. Lectins were also shown to bind to the apical membranes of the gut epithelial cells (EISEMANN et al. 1994). The peritrophic membrane which exists in most phytophagous insects is mainly composed of chitin. It forms a barrier to protect the midgut epithelium from abrasive food particles. Lectins may bind to this membrane and influence movement between peritrophic spaces or prevent formation of the membrane.

GATEHOUSE et al. (1984) demonstrated the binding of a pea lectin to midgut epithelial cells of the bruchid weevil and postulated that the bound lectin may inhibit absorption of nutrients or may disrupt cells by stimulating endocytosis not only of the lectin but also other toxic metabolites and bacterial toxins which are present in the gut. HABIBI et al. (2000) showed that the pea lectin was causing disruption, disorganization and elongation of the border microvilli and swelling of the epithelial cells in the gut lumen, leading to complete closure of the lumen.

Con A lectins were shown to bind mainly to the apical membranes of the stomach epithelial cells (luminal surface) of an aphid species, although at high concentrations

the lectin was shown to be able to bind to the whole epithelium of the digestive system with the exception of the oesophagus (SAUVION et al. 2004). Morphological changes in the epithelial cells such as cellular swelling, hypersecretion and a partial or even complete detachment of epithelial cells resulting in a closure of the gut lumen was observed although the epithelial cells themselves did not seem to be affected (SAUVION et al. 2004). A “hyper-regenerative” effect by the acceleration of the natural turnover of the border membranes of the digestive cells was suggested.

- Binding of lectins to glycosylated digestive enzymes.

Insecticidal effects of lectins can also occur through the interfering of lectins with gut enzymatic functions thus destabilising insect metabolism. This has been shown for **Lacnobia oleracea** larvae in which orally fed GNA and Con A lectins affected soluble and brush border membrane enzymes in the gut and several enzyme activities and levels were increased (see references in VASCONCELOS & OLIVEIRA 2004).

- Binding of lectins to chemoreceptors in the mouthpart.

GATEHOUSE et al. (1995) suggested that the feeding deterrence might be due to the binding of lectins to glyco-proteins on chemoreceptors near the mouthparts of dipteran larvae resulting in partial feeding inhibition. This was also supported by observed avoidance responses of lectins by the larvae as observed by EISEMANN (cited in GATEHOUSE et al. 1995). The hypothesis of reduced food intake due to a feeding deterrent effect being responsible, at least in part, for the adverse effects of lectins was also supported by observations of reduced honeydew production of plant hoppers when feeding on GNA or WGA lectins (see GATEHOUSE et al. 1995). However, recently it has been shown in an aphid model that feeding deterrence was probably a consequence of intoxication and not due to a sensory mediated process (SAUVION et al. 2004).

- Systemic effects.

It was also shown that lectins are able to cross the midgut epithelial barrier, become localised in the cytoplasm of the cells and may result in a systemic toxic effects (see discussion in SAUVION et al. 2004). Internalisation and systemic distribution of other lectins such as GNA and ConA have also been shown in the tomato moth larvae (see references in VASCONCELOS & OLIVEIRA 2004).

8.1.1.1 Sugar specificity of lectins

Plant lectins display differential affinity to monosaccharides to which they bind on cell surfaces. The specificity of lectins to closely related insects does not seem to be related to this monosaccharide specificity. So far, no correlation could be established between the sugar specificity of lectins and their insecticidal activity to aphids (GATEHOUSE et al. 1995, RAHBE et al. 1995). Lectins with specificity to N-acetyl-glucosamine were either non-insecticidal or moderately active to toxic (RAHBE et al. 1995). Also the lectin from **Triticum vulgaris** WGA was non-toxic to the aphid **Acyrtosiphon pisum** but was shown to be toxic to other Homoptera such as planthoppers (see references in RAHBE et al. 1995). Although most of the mannose-binding lectins were toxic to the aphid **A. pisum** other lectins in this class showed low levels of insecticidal activity.

The correlation of sugar specificity and toxicity of lectins to other taxa is also not clear. Only Fabaceae lectins with binding specificities for mannose-glucose residues had no effect on both, Lepidoptera (**Ostrinia nubilalis**) and Coleoptera **Diabrotica undecimpunctata howardi** (CZAPLA & LANG 1990). Lectins with specificity for N-acetylglucosamine or N-acetylgalactosamine/galactose showed no constant insecticidal activity pattern. Studies with a specific coleopteran species showed that different lectins of the same sugar specificity exhibited different insecticidal properties (GATEHOUSE et al. 1995).

Lectins with N-acetyl-glucosamine specificity may be more effective to insects as lectins with other sugar specificities possibly due to the presence of chitin in the peritrophic membrane in the insect's midgut which is a polymer of N-acetyl-glucosamine (GATEHOUSE et al. 1995).

8.1.1.2 Stability of lectins

A main feature of lectins is their marked stability under unfavourable conditions, e.g. over a wide range of pH, heat and their resistance to insect and animal proteases. Only some plant herbivores are capable of digesting lectins (PEUMANS & VAN DAMME 1995). However, the stability of a lectin alone is not sufficient to explain its toxicity (GATEHOUSE et al. 1995).

The resistance of lectins to degradation by proteases might be a common feature of lectins (VASCONCELOS & OLIVEIRA 2004). For their action in the insects gut, lectins have to resist the proteolytic enzymes and encounter satisfactory environmental conditions of pH and temperature. Several lectins have been shown to be resistant to *in vivo* breakdown by proteolytic enzymes although the extent of resistances varies considerably. Far fewer lectins survive the *in vitro* treatment with proteolytic enzymes, being digested to a far higher extent than *in vivo* (VASCONCELOS & OLIVEIRA 2004).

Most lectins pass the gut of aphids without modification or proteolytic degradation while a few others are not excreted (RAHBE et al. 1995). For example, the snowdrop lectin GNA is not significantly degraded either in the gut or in the honeydew of the rice brown planthopper *Nilaparvata lugens* (POWELL et al. 1998 cited in VASCONCELOS & OLIVEIRA 2004). Either non-toxic or highly toxic lectins showed a lack of proteolytic digestion. This observation of a lack of degradation of lectins in the digestive system, irrespective of whether they are toxic or not has also been observed in other orders such as Lepidoptera, although this group has very active digestive proteases (GATEHOUSE et al. 1994 in RAHBE et al. 1995). This apparent absence of degradation of ingested proteins rules out proteolysis as potential detoxification mechanism in aphids as no significant endoprotease activity levels were detected in aphid digestive tracts. Therefore the lack of degradation was considered to be due to the binding of the lectins to the insect gut epithelia (RAHBE et al. 1995). This is supported by the observations of SAUVION et al. (2004) who concluded that the lectin binds to the gut structures of the aphid. Also other authors confirmed that some lectins such as GNA are detectable in honeydew of aphids while others are not, thus indicating that at least some lectins could be transferred to higher trophic levels (see discussion in POWELL 2001).

8.1.2 Ribosome Inactivating Proteins (RIPs): a special class of lectins

Due to the ability of certain toxic proteins to damage ribosomes irreversibly they are also named ribosome inactivating proteins (RIPs). Well-known examples of RIPs are ricin, abrin and modeccin. In contrast to lectins which have multivalent carbohydrate binding sites, these toxic proteins are monovalent being also designated as hemilectins or chimerolectins (CARLINI & GROSSI-DE-SÁ 2002). Two types of RIPs can be distinguished. Type 1 RIPs consist of single polypeptide chains while those existing as two disulphide-linked polypeptides are type 2 RIPs (STIRPE & BARBIERI 1986 cited in GATEHOUSE et al. 1990). The majority of RIPs are single-chain proteins and only few type 2 RIPs have been described so far, such as modeccin, volkensin, viscumin, ebulin or nigrin.

Type 2 RIPs consist of an enzymatically active A-chain and a slightly larger carbohydrate binding B-chain with sugar-binding specificity, the lectin moiety. The B-chain recognizes the target cell by binding to a glyco-conjugate receptor on the cell surface thus enabling the uptake of the A-chain. After cell entry the A-chain catalytically

inactivates eukaryotic ribosomes by cleaving the N-glycosidic bond of a single adenosine residue of the 28S rRNA. The modified ribosomes are thus unable to support protein synthesis (see review of OLSNES 2004). One molecule is able to inactivate a few thousand ribosomes per minute. In the enzymatically active A-chain a number of highly conserved residues, located in the active sites, are important and mutation of one of these strongly reduces the enzymatic activity of the chain. These amino acids are conserved throughout the RIP protein family and are decisive for the enzymatic effect (see review of OLSNES 2004).

In contrast type 1 RIPs consist only of a single polypeptide which corresponds to the enzymatically active A-chain polypeptide but they are unable to enter intact cells as efficiently as the type 2 RIPs as they lack the lectin moiety (GATEHOUSE et al. 1990). The B-chain lectin polypeptide is considered necessary for toxicity as it binds to specific sites on the cell surfaces thereby effecting uptake and internalisation of the toxic polypeptide chain (see GATEHOUSE et al. 1990). This is also supported by the fact that ricin, a type 2 RIP with a lectin moiety shows toxicity towards the aphid *A. pisum* while type 1 RIPs do not exhibit any toxicity (RAHBE et al. 1995). Type 1 RIPs can become highly toxic if they are linked to an appropriate carrier capable of binding to cells (STIRPE 2004).

8.1.3 Enzyme inhibitors

8.1.3.1 Alpha-Amylase Inhibitors (alpha-AIs)

Inhibition of alpha-amylases by many alpha-AIs (lectin-like, cereal-type, knottin-type alpha-AIs) occurs through formation of an inhibitor-enzyme complex. This complex blocks the catalytic site thus blocking the binding of the enzyme to the substrate ("competitive inhibition"). In contrast Kunitz-like α -AIs have a different mode of action as they do not interact directly with the catalytic residues of the enzyme but with domains near the catalytic site and thereby preventing substrate access. In other cases the exact mode of action is still not resolved (e.g. antifungal mode of thaumatin-like α -AIs) or the mechanisms vary considerably including the modification of the membrane permeability, inhibition of protein synthesis or proteinase inhibition.

Inhibitors can have two independent inhibition sites with the N-terminal site being responsible for alpha-AI inhibition while at the opposite site a trypsin inhibitor region is present (e.g. Kunitz-like or cereal-type α -AIs).

8.1.3.2 Proteinase Inhibitors (PIs)

Most serine PI families and probably to the cysteine and aspartyl PI families are competitive inhibitors inhibiting proteinases with a similar mechanism as proposed by LASKOWSKI & KATO (cited in HAQ et al. 2004). A binding between the reactive site on the surface of the inhibitors and the binding region of a proteinase leads to the inhibition of the respective proteinase. The interaction is highly specific as the peptide bond specifically interacts with the active site of the enzyme. These complexes with digestive proteases are highly stable and dissociate very slowly.

In phytophagous insects the two major proteinase classes in the digestive system are serine and cysteine proteinases. Serine proteinases are dominant in lepidopteran guts. Coleoptera have a wider range of dominant gut proteinases (see HAQ et al. 2004). In insects trypsin is also involved in developmental processes such as molting, therefore disruption of these processes by PIs is also considered possible.

Originally it was proposed that reduction or inactivation of gut proteolysis and consequently poor nutrient utilisation lead to retarded development and eventual death by starvation of a herbivore feeding on a PI-producing plant (GATEHOUSE et al. 1999). Later it was suggested that a hyperproduction of proteinases to compensate for the loss of activity lead to the depletion of essential amino acids and consequently to growth

retardation (HAQ et al. 2004). OPPERT et al. (1993) suggested that both, cysteine and serine PIs cause amino acid deficiencies that lead to detrimental physiological effects. In their experiments they showed that negative effects of PIs on larval growth of a coleopteran species were to some extent reduced when supplemented by the addition of an amino acid mixture to the diet. Similar suggestions were made for serine PIs and Lepidoptera when supplemented with methionine. Therefore the mechanism by which proteinase inhibitors slow growth and cause mortality was suggested to be similar and to involve a deficiency in one or more essential amino acids (OPPERT et al. 1993).

The effect of soybean trypsin inhibitor against larval Lepidoptera was originally considered to be the competitive binding of a PI to midgut proteolytic enzymes and the lessening of the rate of proteolysis in the gut of an insect. However, it has been shown that the growth reducing effect was due to a toxic rather than an antidiigestive effect (DUFFEY & STOUT 1996). The presence of soybean PI did not alter the proteolysis of a protein but supplemented growth in proportion to the added protein content of the proteinase suggesting that these PIs acted as nutrients rather than as "antinutrients" (DUFFEY & STOUT 1996). There are indications that these PIs show toxicity towards the larvae as they induce trypsin biosynthesis and induce a loss of amino acids to make tryptic proteases rather than body proteins. At higher levels of the PI the nutritional gain of the PI is outweighed by its toxic effects thus leading to larval growth reduction (DUFFEY & STOUT 1996).

Factors affecting inhibitory activity of PIs

Additive effects of different factors (e.g. low quality of protein diet and the presence of reactive enzyme) on an insect's growth have been highlighted by DUFFEY & STOUT (1996). They argued that the activity of PIs is influenced by both, the quantity and the quality of dietary protein. The growth reducing effect of the PI can be dependent on the nutritional value of the larvae's diet. When protein was provided at optimal quantity the effect of the PI to an insect was minimal. Also methionine, arginine and lysine contents of the dietary protein influenced the effect. The addition of the amino acid cysteine to the diet reduced the impact of the soybean PI on the insect (DUFFEY & STOUT 1996).

Also the presence of other chemical factors such as reducing agents (e.g. thiols) and alkylators affect the effectiveness of PIs. This is explained by the effect of these factors on the disulphide bonds which are necessary for the activity of the PI. As has been shown, the chemical integrity and consequently the biological activity of PIs can be altered not only by proteins and reducing agents but also by reactive organic or inorganic factors produced by enzymes such as oxidase, peroxidase and lipoxygenase and other chemical or enzymatic oxidative systems (DUFFEY & STOUT 1996).

The pH and redox potential of the digestive tract of an insect also influences the efficacy of a PI (DUFFEY & STOUT 1996). The high pH observed in lepidopteran digestive fluids might oxidatively damage disulphide bonds. The pH and the type of the insects proteolytic enzymes are correlated. Lepidopteran larvae have a basic gut pH and rely mostly on serine proteases as major digestive enzymes (trypsins, chymotrypsins and elastases) which themselves have a high pH optimum. In contrast, coleopteran larvae with acidic gut pH rely on thiol-based proteinases which would be chemically unstable at high pH. Many coleopteran species have cysteine proteinases as their major digestive enzymes (see references and discussion in OPPERT et al. 1993).

8.2 Fungal proteins

Fungal toxins have been shown to exert toxic effects on individual cells, inhibiting cell growth, alternating cell morphology (LIU et al. 1996) or reducing metabolic activity (QUESADA-MORAGA & VEY 2004). Fungal toxins also possess insecticidal properties against various insects and can induce increased mortality in some insect species (QUESADA-MORAGA & VEY 2004). GANASSI et al. (2002) have shown that Beauvericin even has

reproductive toxicity on an aphid species, as it was shown not to affect survival of the aphids but reducing the number of offspring in the third generation and inducing abort of embryos ingested by aphids. As a possible mechanism the authors suggested the interaction of the fungal toxin or its derivatives and nucleic acids of aphid bacterial symbionts.

Hirsutellin A is a potent inhibitor of protein synthesis and causes specific cleavage of ribosomal RNA, thus inhibiting insect cell growth (LIU et al. 1996). It has been suggested that Hirsutellin A is specific to invertebrates, as invertebrate cells possess appropriate receptors or transport mechanism or lack the detoxification mechanisms as this toxic protein did not cause detectable cytopathic effects on green monkey kidney cells or kill mice (LIU et al. 1996).

The exact mechanism of Bassiacridin is still unknown. It has been suggested that Bassiacridin can directly or indirectly activate the prophenoloxdase in the cuticle of the tracheae and air sacs of host organisms and that the toxic protein acts via chitin-binding properties (QUESADA-MORAGA & VEY 2004).

8.3 Cyanobacterial and bacterial proteins

8.3.1 Cyanobacterial proteins

The mode of action of Microcystins and Nodularin is a specific inhibition of protein phosphatases type 1 and 2A. The amino acid **Adda** and its structure are playing a crucial role for the inactivation of these enzymes (WIEGAND & PFLUGMACHER 2005). The mode of action of Nodularin is very similar to that of the Microcystin. The type of variant of Microcystins plays a crucial role for their effectiveness on zooplankton species (WIEGAND & PFLUGMACHER 2005).

8.3.2 Bacterial proteins

Bacterial proteins can be divided into two classes, toxins that act intracellularly and toxins that act at the cell surface. Mechanisms of intracellularly acting bacterial toxins include elevating intracellular cyclic AMP (e.g. cholera toxin) or blocking protein synthesis (e.g. diphtheria toxin). However, these toxins need to possess mechanisms to penetrate a cell before they can exert their toxic action. These proteins are composed of two chains, each with a separate function. While one fragment is responsible for the enzymatic action, the other fragment contains two domains, one involved in receptor binding and the other involved in the formation of channels in cell membranes (for review see ENGLISH & SLATIN 1992).

The second class of bacterial toxins contain proteins which, dependent on the pH, form channels that themselves damage the cell by disrupting the osmotic balance. The cytolytins are a family belonging to this group of channel-forming toxins, but also the ***Staphylococcus aureus*** alpha-toxin (ENGLISH & SLATIN 1992). The surface-acting colicins produced by ***E. coli*** also form voltage and pH-dependent channels. These proteins are generally composed of three domains, a receptor-binding domain, a translocation-domain near the N-terminal and an ion-channel forming domain at the C-terminal. Interestingly, receptor binding is not a prerequisite for channel formation (ENGLISH & SLATIN 1992). Also endotoxins of ***Bacillus thuringiensis*** are considered channel-forming toxins.

8.3.2.1 Endotoxins of *Bacillus thuringiensis*

The Cry- and the Cyt-toxins produced by ***Bacillus thuringiensis*** are pore-forming toxins. These proteins undergo conformational changes in order to insert into or translocate

across cell membranes of their hosts. After secretion of the toxins by bacteria, these toxins interact with specific receptors located on the host cell surface. Typically, the toxins are activated by host proteases after receptor binding (BRAVO et al. 2007). The Cry toxins differ in their pore forming mechanism from the Cyt toxins (BRAVO et al. 2007).

The larger group of the Cry-toxins (Cry1, Cry2 and Cry3: 3-domain family) contains three structural domains. Its main characteristic is the presence of protoxins with different lengths. The tertiary structures of six different Cry proteins have been elucidated (Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba). These show a high degree of similarity suggesting a similar mode of action within the Cry-3-domain family (BRAVO et al. 2007). Domain I (N-terminal) is responsible for membrane insertion and pore formation. Domains II and III are involved in receptor binding. Domain II and particularly domain III have structural similarities with carbohydrate-binding proteins such as lectins suggesting that the carbohydrate moiety could be important for the mode of action of these toxins and consequently for their specificities (see reviews in BRAVO et al. 2007 and PIGOTT & ELLAR 2007). Further analysis has shown that swapping of domain III among toxins has occurred and that novel specificities occur (BRAVO et al. 2007).

The primary mode of action of Cry toxins is the forming of pores in the apical microvilli membrane of the midgut epithelial cells of the target insect (BRAVO et al. 2007). The crystal inclusions are ingested by susceptible insects and dissolve in the alkaline environment of the gut releasing one or more delta-endotoxins. The solubilized inactive protoxins are cleaved by midgut proteases resulting in protease-resistant proteins with the proteolytic removal of an N-terminal peptide and part of the C-terminus. The activated protein then binds to the receptors on the brush border membrane of the midgut epithelium columnar cells and inserts into the membrane leading to the formation of pores in microvilli of the membranes. Subsequently, cell lysis and the disruption of the midgut epithelium occurs (BRAVO et al. 2007).

Receptor binding of the Cry-toxin to the insect midgut epithelium is considered an important determinant of specificity. Reversible binding correlates with toxin binding to receptors while irreversible binding was associated with membrane insertion (PIGOTT & ELLAR 2007). Several different receptor molecules have been described for various δ -endotoxins in different insects. Also the resistance mechanism to Cry toxins has been considered due to changes in receptor binding (FERRE & VAN RIE 2002 cited in BRAVO et al. 2007). Aminopeptidase-N proteins and cadherin-like receptors have been identified but also other receptors such as glycolipids or alkaline phosphatases were recently described (PIGOTT & ELLAR 2007).

8.3.2.2 Other bacterial toxins

One class of toxins of the bacterial genera *Photorhabdus* and *Xenorhabdus* are commonly referred to as “toxin complexes”. They have been also documented in a range of bacteria, some related to insects while others with no obvious link to insects. These bacterial toxins exhibit toxicity to caterpillar pests after ingestion (BOWEN et al. 1998 cited in WATERFIELD et al. 2005).

Toxin complexes are composed of sub-complexes which are classified into A, B and C components. The presence of multiple copies of *tc* genes suggests that a mixture of toxins are employed by the bacteria to kill their hosts. Additionally, it has been shown that different A components of Tc's have different activity against different caterpillars, suggesting that additional copies of A components provide a mixture of toxins active against a range of insects. Although the A component alone is a toxin, the B and C components act as toxin “potentiators” as full toxicity is only restored only when these components are also expressed. Also “cross-potentiation” has been shown, as one

component pair (BC) can potentiate different A toxins (WATERFIELD et al. 2005, FFRENCH-CONSTANT et al. 2007).

A second class of bacterial toxins are called “**Photorhabdus** insect related” (Pir) proteins which are encoded by the genes PirA and PirB. Pir B has been shown to be similar to the pore-forming domain of **Bt** Cry toxins (FFRENCH-CONSTANT et al. 2007).

However, the details of the modes of action of various toxins of **Xenorhabdus** and **Photorhabdus** bacteria are still unknown. The A component was shown to disrupt the insect midgut epithelium in a similar manner to the delta-endotoxins from **Bt**. Ingestion by lepidopteran larvae has been shown to damage the midgut cells, leading to epithelium shed into the gut lumen followed by epithelium lysis, but not damaging other tissues of lepidopteran larvae (BLACKBURN et al. 1998 cited in BROWN et al. 2006).

Recently, membrane binding studies with the toxin XptA1 from **Xenorhabdus nematophila** have shown that the toxin first binds to the cell membrane and then forms a complex with the other two components to reach full insecticidal toxicity (LEE et al. 2007). As the toxin is stable at high pH values it can pass intact and without further processing through the insects gut before interacting with the target membranes which is different from the mode of action employed by **Bt** proteins (LEE et al. 2007).

9 Ecotoxicity data on proteins

9.1 Plant proteins

9.1.1 Lectins

Most of the reported studies on effects of lectins on insects have been described for phytophagous insects. Studies using dipteran species are rare, possibly because they play a relatively minor role in crop damage. According to GATEHOUSE et al. (1995) conventional insecticides used against Diptera account only for 3 % of total world insecticide usage.

Most studies on lectin toxicity deal with agronomically relevant insect species, especially if they have pest status. For example, studies concentrate on important coleopteran pests such as the cowpea weevil *Callosobruchus maculatus* which attacks dried cowpeas and other related stored seeds or the Southern corn rootworm *Diabrotica undecimpunctata* whose larvae feed on roots of a wide range of plants, including many field crops. Also within the order of Homoptera most studies deal with agronomically important species such as the cabbage aphid or the potato aphid. Plant and leafhoppers such as the taro planthopper or the brown planthopper which cause significant damage to crop plants are preferentially considered.

Lectins with similar monosaccharide specificity display different effects on closely related insects. For example, the response of one aphid species to various lectins differed significantly (RAHBE et al. 1995). The authors grouped the lectins into three groups according to their toxicity:

- The majority of proteins impaired growth or nymphal survival only slightly (e.g. lectins of *Vicia villosa*, *Triticum vulgaris* or *Bryonia dioica*).
- A small number of proteins caused significant mortality at the highest dose (lentil, snowdrop, amaranth, jack bean lectins).
- Other proteins were less toxic but inhibited significantly growth (lectins of *Bandeira simplicifolia*, *Sambucus nigra* and *Pisum sativum*).

A specific lectin can have very differential effects on different species. The lectin of *Canavalia ensiformis* (Con A) was tested on several aphid species for its insecticidal activity over a range of concentrations (RAHBE et al. 1995). At high concentrations all species were affected and showed growth inhibition. More sensitive species (*A. pisum* and *M. albifrons*) were unable to survive on concentrations higher than 750 µg/ml with adverse responses also at lower concentrations (< 100 µg/ml) by *A. pisum*. Other species tolerated higher concentrations. Two distinct response groups were distinguished. Some species did not show an immediate effect but responded to the toxin at higher concentrations. In contrast other species responded immediately to the toxin showing growth retardation and then again showed a response at higher concentrations. The species could not be grouped according to their taxonomic position but rather according to their host range characteristics. Aphid species restricted to the plant family of the Fabaceae were the most sensitive while polyphagous aphids displayed greater resistance to the lectin (RAHBE et al. 1995).

Plant-derived lectins can exhibit different toxicity profiles. When tested on aphids concanavalin A (Con A) showed no acute toxicity at low doses but was a potent growth inhibitor. In contrast wheat germ agglutinin (WGA) was slightly toxic and showed no dose response (RAHBE & FEBVAY 1993).

The *Pisum sativum* plant lectin was shown to be toxic for some species of Lepidoptera or Coleoptera but not for others (BOULTER et al. 1990, CZAPLA & LANG 1990). The lectin from

the pea *Pisum sativum* was toxic to the bruchid weevil *Callosobruchus maculatus* but it was ineffective against the Southern corn rootworm *Diabrotica undecimpunctata* (GATEHOUSE et al. 1995).

Three plant lectins (*Ricinus communis*; Leguminosae; *Triticum vulgaris*; Poaceae; *Bauhinia purpurea*; Caesalpiniaceae) were toxic to the European corn borer (*Ostrinia nubilalis*; Lepidoptera). The first two also exhibited significant weight gain inhibition. Other lectins tested did not affect larval growth or mortality. Some lectins even increased larval weight by > 25 % (soybean lectin *Glycine max*). In contrast, in another study the same lectin significantly affected lepidopteran larval growth (GATEHOUSE et al. 1995).

For the corn rootworm (*Diabrotica undecimpunctata howardi*; Coleoptera) lectins of *Ricinus communis* (Leguminosae), *Phytolacca americana* (Phytolaccaceae) and *Codium fragile* (Codiaceae, marine algae) were toxic while significant growth inhibition was shown for lectins of *Maclura pomifera* and *Artocarpus integrifolia* (both Moraceae), *Vicia villosa* (Leguminosae), *Bandeiraea simplicifolia* (Caesalpiniaceae), *Triticum vulgaris* (Poaceae) and also *Codium fragile* (Codiaceae, marine algae; CZAPLA & LANG 1990).

9.1.2 Ribosome inactivating proteins (RIPs)

Type 2 RIPs are considered highly toxic to all eukaryotes, but toxicity of ribosome inactivating proteins in general is reported to be highly variable. While the RIP from castor bean seeds (*Ricinus communis*), ricin, is highly cytotoxic, RIPs from edible grains such as wheat or barley generally exhibit no toxicity (see review in DOWD et al. 1998).

The RIPs ricin and saporin are toxic to coleopteran larvae but non-toxic to lepidopteran larvae (GATEHOUSE et al. 1990). The effectiveness of both toxins differed approximately 10fold between two coleopteran species tested. Ricin was the most potent toxin towards the coleopteran pest *C. maculatus* while saporin was 10fold less effective. Both toxins were also slightly less toxic towards the coleopteran species *A. grandis*. In contrast, no adverse effects of these toxins were observed for two lepidopteran insects. The observed lack of toxicity to Lepidoptera was explained by the presence of serine proteases which hydrolyse the toxins while coleopteran larvae do not possess these gut enzymes. The proteins were hydrolysed in lepidopteran gut enzymes while they remained intact when incubated in coleopteran gut enzymes.

DOWD et al. (1998) have shown that the activated form of the maize RIP was active against beetles and caterpillars at the same concentrations but the susceptibility of the species varied considerably. The authors suggested that the susceptibility to the maize endosperm RIP can be explained rather by host adaptation, with those species normally not feeding on maize being most severely affected (DOWD et al. 1998).

9.1.3 Alpha-Amylase Inhibitors

The specificity of alpha-amylase inhibitors may vary considerably. Different α -amylase inhibitors exhibit different specificities against alpha-amylases from diverse sources. Some alpha-AIs only act against mammalian others against insect α -amylases. In general alpha-AIs inhibit several α -amylases from different sources. For example the alpha-AI of wheat WRP26 is unable to inhibit the bean weevil *Zabrotes subfasciatus* (Coleoptera, Bruchidae) while WRP25, having a 98 % identical sequence to WRP 26, effectively inhibits this species. Similarly, two alpha-AIs of the common bean *Phaseolus vulgaris* exhibit differential specificities towards this coleopteran species. While alpha-AI1 does not inhibit this species, alpha-AI2 does (FRANCO et al. 2002).

In the gut lumen of some insect species different forms of alpha-amylases can be observed. Also patterns of expression vary when an insect is fed on different diets. This

has been interpreted as a response to the occurrence of inhibitors in the diet. Some weevils even have the ability to modulate the concentration of these proteins when fed different diets. This diversity of alpha-amylases in the insect's gut could explain the low *in vivo* efficiency of some inhibitors against insect pests. A certain alpha-AI would need to have a very broad specificity, inhibiting all the alpha-amylases present in the insect's gut.

Also *in vivo* conditions can influence alpha-amylase specificity. Certain alpha-amylases are well adapted to the slightly acidic physiological environment of the larval midgut (pH optimum of 5.8 for the cleavage of starch). Similarly, the acidic optimum pH for inhibition by the bean α -AI may be responsible for their inhibition of amylases in Coleoptera but not in Lepidoptera with an alkaline gut milieu.

Explanations of differences in specificity between alpha-AIs include size and electrostatic properties of key residues, in some cases different loop lengths in α -amylases of insects or mammals, conformational differences resulting from the presence of certain amino acids at certain positions, steric factors, electrostatic properties, hydrogen bonding capability, the special conformational properties of proline and the disulfide bonding of cysteine and even multiple factors.

9.1.4 Proteinase inhibitors (PIs)

PIs are known to be effective against nematodes, fungal infections and viruses (see review in Haq et al. 2004). The effectiveness of proteinase inhibitors has been examined for several lepidopteran, coleopteran, orthopteran and dipteran insects (POMPERMAYER et al. 2001, OPPERT et al. 1993). In most cases pest species such as ***Callosobruchus maculatus***, ***Helicoverpa armigera***, ***Heliothis virescens*** or different aphid species are examined in order to determine whether they can be controlled by the use of proteinase inhibitors.

Serine PIs were shown to be effective against Lepidoptera whereas cysteine PIs were shown effective against some Coleoptera. Aphids were affected by cysteine-type PIs as these types of proteinases form the major endoproteolytic system in the aphid digestive tract. A Bowman-Birk inhibitor was also shown to be effective in aphids.

Serine proteinase inhibitors (SPIs) lead to retarded growth of several lepidopteran, coleopteran, orthopteran and dipteran insects (see references in POMPERMAYER et al. 2001 and OPPERT et al. 1993) although in some cases they exhibited rather poor efficacy. Bowman Birk inhibitors from soybean are effective growth inhibitors for coleopteran and lepidopteran larvae. Kunitz inhibitor from soybean (SBTI) inhibits the growth of lepidopteran and coleopteran larvae (SHEWRY & LUCAS 1997). However, in Lepidoptera the effectiveness of the soybean Kunitz inhibitor has given variable results. This inhibitor reduced larval growth and survival of the lepidopteran pest ***Helicoverpa armigera*** but not to the closely related ***Heliothis virescens*** (GATEHOUSE et al. 1999).

The studies of BROADWAY & VILLANI (1995) showed that several trypsin inhibitors inhibited the proteolytic activity *in vitro* of several coleopteran insect species while a chymotrypsin inhibitor had limited inhibitory activity. The Kunitz soybean PI significantly affected proteolytic activity in two coleopteran and one lepidopteran larvae while it had no effect on proteolytic activity of three other coleopteran larvae. This study also showed that not all species for which digestive enzymes can be blocked *in vitro*, are actually susceptible to the PIs *in vivo*. This is explained with the insect's ability to secrete additional trypsins in response to the trypsin inhibitor ingested and these trypsins have different susceptibility to PIs (see discussion in BROADWAY & VILLANI 1995). The authors suggest that the susceptibility of certain species to the PIs may be determined by the host range of the individual species because species with generalized feeding habits experience none or only small effects on the proteolytic activity in their

midguts while specialised species were particularly affected. This susceptibility based on host range might be explained by a shift in proteolytic enzymes and to the presence of “resistant” enzymes in their midgut of the generalists. A resistance to a specific PI may also mean a resistance to many PIs within a specific PI family such as the PIs from monocots, including corn, millet and barley. Thus an insect feeding on corn may be resistant to PIs in millet and barley, even without prior exposure because these PI groups are based on sequence similarity, topological similarity and mechanism of binding. Therefore if generalists encounter non-host PIs they are better adapted to secrete resistant proteinases due to prior exposure to an inhibitor from the same family while the specialist is less likely to have experienced a PI from this non-host PI family.

Also other authors suggested that generalist feeders are better adapted to deal with PIs than specialists as PI insensitive proteinases are probably the result of selection pressures when insects are exposed to high PI levels in their host plant which has not occurred in non-host plant PIs (see references in POMPERMAYER et al. 2001). As responses to chronic ingestion of PIs it has been shown that lepidopterans can either stimulate endogenous proteolytic activity or induce production of proteases which are resistant or insensitive to PIs (GATEHOUSE et al. 1999).

TRAN et al. (1997) screened five purified PIs against three important cereal aphid species and found that their effectiveness varied greatly with aphid species. For example a carboxypeptidase inhibitor was effective only against *Rhopalosiphum padi* and not against the two other aphid species tested while potato proteinase inhibitors I and II were effective against all three species tested. The same effects were observed for the production of nymphs. The proteinase inhibitors effectively blocked nymph production of all three cereal aphids tested.

Even though effectiveness of PIs has been described for many insect species their specificities cannot be assumed *a priori* as the effectiveness varies between PIs and depends on the species tested. There is evidence that certain proteinase inhibitors have wider specificities than originally thought (see overview in SHEWRY & LUCAS 1997). Additionally, insects have the ability to develop strategies to deal with the adverse effects of PIs on their digestive system such as the degradation of PIs by non-target proteinases or the induction and upregulation of other proteinases that are not inhibited (CARLINI & GROSSI-DE-SÁ 2002).

9.2 Fungal proteins

Adverse effects of beauvericin, a toxic fungal protein, on aphid offspring although not affecting survival were observed by GANASSI et al. (2002). The effects were only observed in the second and third generation. The authors suggested an interaction between beauvericin or its derivatives and the nucleic acids of prokaryotic endosymbionts of the aphids contained in specialized cells, the bacteriocytes, thereby affecting fecundity. The toxin effects already the first generation but is only detected in the second or third generation. Another explanation is that only the third generation embryos, have a higher sensitivity against the toxin already present in larvae of the first generation (GANASSI et al. 2002).

9.3 Bacterial proteins

The classification of the crystal *Bt* toxins was originally done by HÖFTE & WHITELEY (1989). They defined four major classes by their spectrum of activity against Lepidoptera (CryI), both Lepidoptera and Diptera (CryII), Coleoptera (CryIII) and Diptera (CryIV). Later CryV toxins were described with activity against Lepidoptera and Coleoptera and further delta-endotoxins with activities against Hymenoptera, Coleoptera and nematodes (KOZIEL et al. 1993). The spectrum of insecticidal activity of an

individual delta-endotoxin is considered to be quite narrow with a certain toxin being active against only a few target pest insects. It has been recognized that the limited specificity might be also a result of the limited testing that these proteins undergo in order to identify their activity as typically these proteins are tested on only a few pest insect species of commercial relevance (KOZIEL et al. 1993).

MACINTOSH et al. (1990) established the host range and efficacy of purified **Bt** proteins, CryIIIA (now Cry3A), CryIA(b) (now Cry1Ab) and CryIA(c) (now Cry1Ac), by screening seventeen agronomically important insect species and one mite species using bioassays and establishing LC₅₀ and EC₅₀ values. The relative sensitivity of lepidopteran larvae to the Cry1A proteins varied significantly and mortality of the larvae showed a very different dose-response curve. In some lepidopteran species sublethal doses caused reduction in larval growth at very low protein concentrations. While for some species the EC₅₀ was 10fold below their LC₅₀ for other species the EC₅₀ were 500–1000fold below the respective LC₅₀ values. The only insect that was sensitive to the Cry3A protein was the Colorado potato beetle (***Leptinotarsa decemlineata***). The overall activity range of the Cry1Ab protein across seven sensitive lepidopterans was approximately 2000fold (MACINTOSH et al. 1990).

SLANEY et al. (1992) tested the CryIIIA protein (now: Cry3A) against two coleopteran species of the Chrysomelidae, the Colorado potato beetle, ***Leptinotarsa decemlineata***, and the Southern Corn rootworm, ***Diabrotica undecimpunctata howardii***. They found that the former species was at least 2000fold more sensitive to this Cry-toxin than the latter. As an explanation the authors suggested reduction in the binding of the toxin to the membrane receptors and in membrane permeability induced by the toxin.

When isolated, the Cry1F protein was tested against four neonate lepidopteran insects (***Heliothis virescens***, ***Helicoverpa zea***, ***Spodoptera exigua*** and ***Ostrinia nubilalis***) for its insecticidal activity (CHAMBERS et al. 1991). The Cry1F toxin was highly toxic for ***O. nubilalis*** and ***H. virescens*** but moderately active against ***S. exigua*** and exhibited little toxicity against ***H. zea***. In comparison with the Cry1Ab toxin the Cry1F toxin was more toxic for ***H. virescens*** and ***S. exigua***, significantly less toxic to ***H. zea*** and equally toxic against ***O. nubilalis***. Compared with the Cry1Ac toxin, the Cry1F toxin was significantly more toxic to ***S. exigua*** but significantly less toxic to ***H. virescens***, ***H. zea*** and ***O. nubilalis***.

Binary insecticidal crystal proteins composed of a 14 kDa (Cry34Ab1) and a 44kDa (Cry35Ab1) protein showed activity against the Western corn rootworm ***Diabrotica virgifera virgifera*** (NARVA et al. 2000a, 2000b cited in HERMAN et al. 2002). Either individually or in mixtures it was shown that the 44 kDa protein did not induce any mortality in the Southern corn rootworm, but even promoted insect growth although the authors explained this effect by an artefact of the bioassay method (HERMAN et al. 2002). In contrast, the 14 kDa protein induced growth inhibition and mortality (HERMAN et al. 2002). Synergism between the two toxins was observed for growth inhibition and mortality. Generally, growth inhibitory effects were observed at much lower concentrations than mortality (HERMAN et al. 2002).

10 Ecotoxicity assessment of microbial plant protection products

Plant protection products are regulated according to Commission Directive 2001/36/EC (EUROPEAN COMMISSION 2001a) amending Directive 91/414/EC (EUROPEAN COUNCIL 1991) concerning the placing of plant protection products on the market. According to Directive 91/414/EC active substances include chemical products but also micro-organisms, having general or specific action against harmful organisms or on plants, part of plants or plant products (Directive 91/414/EC, Article 2). Micro-organisms are defined as microbiological entities, cellular or non-cellular, capable of replication or of transferring genetic material. Substances or preparations including *Bacillus thuringiensis* to be used as or in plant protection products are therefore covered by this Directive.

For the authorization of a plant protection product the application has to contain a dossier satisfying the requirements set out in Annex III and for each active substance the requirements set out in Annex II. Annex II is separated into part A dealing with chemical substances while part B provides data requirements for active substances consisting of micro-organisms including viruses. If the plant protection action is known to be due to a residual effect of a toxin or metabolite or if significant residues of toxins or metabolites are to be expected which are not related to the effect of the active substance, a dossier for the toxin or metabolite in accordance with the requirements for chemical substances (Annex IIA) has also to be submitted.

10.1 Approach of the risk assessment of effects on non-target organisms

Annex IIB specifies the data requirements for the active ingredient with respect to effects on non-target organisms. The characterisation and identification of a micro-organism forms the basis of the assessment. However, data derived from toxicological and/or pathological experiments on laboratory animals are normally required unless the applicant can justify that the use of the micro-organisms does not have any unacceptable influence on the environment.

Equivalence of the micro-organisms used for the testing and the micro-organisms used in the manufacture of preparations is considered crucial and must be established. A detailed description of the material used and its impurities must also be provided.

10.2 Assessment of the fate and behaviour in the environment

According to Annex IIB the information on the origin, the properties and the survival of the micro-organism and its residual metabolites and its intended use form the basis for the assessment on the fate and behaviour in the environment. Experimental data are usually required unless it can be justified that an assessment can be performed with the information already available. Annex IIB refers to a separate assessment under Annex IIA for any relevant metabolites if certain conditions are met (stability of the metabolite, toxic effect of the metabolite is independent of micro-organism, metabolite concentrations higher than under natural conditions).

Information on the persistence and multiplication of the micro-organism in all environmental compartments has to be given unless it can be justified that exposure of the particular environmental compartment to the micro-organism is unlikely to occur. Estimated levels of the micro-organism in a time course after the use of the product

shall be given. Environmental compartments to be considered are soil, water and air. Information on soil should include several cultivated and uncultivated soils representative of soils typical of the various Community regions where use exists or is anticipated.

Requirements for the assessment of the mobility including the possible spread of the micro-organism and its degradation products in relevant environmental compartments have also to be met if relevant.

10.3 Assessment of effects on non-target organisms

Annex IIB outlines the necessary information on identity, biological properties as well as on fate and behaviour of the respective micro-organism which is central to the assessment of the impact on non-target species. Experimental data for the evaluation of effects on non-target organisms are normally required unless it can be justified that an assessment can be performed with the information already available. Tests must be performed unless it can be justified that the non-target organism will not be exposed to the micro-organism.

The choice of the appropriate non-target organism for testing of environmental effects should be based on the identity of the micro-organism including host specificity, mode of action and ecology of the organism. Annex IIB also emphasizes the need to report all potentially adverse effects found during routine investigations and all available biological data and information which is relevant to the assessment of the ecology profile of the micro-organism.

Separate studies may be required for relevant metabolites, especially toxins, where these products either can constitute a risk to non-target organisms or where their effects cannot be evaluated by the available results.

Data requirements including information on toxicity, infectiveness and pathogenicity are required for:

- Wildlife vertebrates (birds and mammals).
- Aquatic organisms (fish, freshwater invertebrates and algae).
- Bees and other beneficial arthropods.
- Soil organisms (earthworms, macro-organisms, micro-organisms).
- Other non-target organisms (e.g. non-target flora).

The selection of test species for non-target arthropods should be related to the potential use of the product, e.g. foliar or soil application. Special attention should be given to organisms used for biological control and those playing an important role in integrated pest management.

Further studies may be required which may include acute studies on additional species or processes or higher tier studies (see next Chapter) such as chronic, sub-lethal or reproductive studies on selected non-target organisms. Higher tier studies are considered necessary if a high risk is identified in the first tier risk assessment or due to special characteristics of the micro-organism such as high persistence or a specific mode of action.

10.4 Evaluation and decision making

Annex VI of Directive 91/414/EC lays down uniform and specific principles of the evaluation and the decision making of a microbial plant protection product.

The possibility of the plant protection product reaching different environmental compartments such as soil, ground and surface water as well as the air under the proposed conditions of use has to be considered as laid down in Annex VI. If a possibility exists that the product reaches the soil then the rate and the route of degradation in the soil, the mobility in the soil and the change in the total concentration of the active substance and of relevant metabolites, degradation and reaction products shall be estimated. The evaluation will consider the specific information on fate and behaviour in soil as provided for in Annex II and other relevant information on the active substance such as photodegradation rate, water solubility or hydrolysis rate. For surface waters a suitable calculation model should be used to estimate the short-term and long-term predicted concentration of the active substance and of metabolites, degradation and reaction products. If such a model is not available, the evaluation should be based on the results of the mobility and persistence in soil and information on run-off and drift.

The evaluation of effects on non-target organisms is based on potential effects on wild living organisms that may be caused by the micro-organism.

Estimated exposure levels must be taken into account and play a key role in the evaluation strategy also considering normal levels of exposure to micro-organisms in the environment. These aspects are also relevant for metabolites and toxins. Exposure assessment should include the survival of the micro-organism in the respective compartment, its ecological niche, the natural background level, information on fate and behaviour in the environmental parts and – if relevant – other uses of the product in the area of envisaged use of the same organism.

The evaluations for the non-target organisms should take into consideration the specific information on the toxicity to non-target organisms as provided for in Annexes II or III and other relevant information (e.g. photodegradation, solubility in water etc.).

The evaluation for a specific non-target organism will include the toxicity/exposure ratio as well as other information including the fate and distribution of the active substance, relevant metabolites, breakdown and reaction products in water sediments or fish or in various parts of the environment (for vertebrates and aquatic organisms) and if relevant, the bioconcentration and persistences of residues in the organism as well as lethal and sublethal effects (for earthworms and non-target soil macro-organisms).

Central to the evaluation is the calculation of toxicity/exposure ratios or hazard quotients based on the quotient of the respective toxicological endpoint tested as well as the estimated exposure. Relevant endpoints to be used are LD₅₀, LC₅₀, EC₅₀, ER₅₀ and NOEC. Triggers for the authorisation of a microbial plant protection product are outlined in Annex VI including triggers for acute and long-term toxicity for terrestrial vertebrates, aquatic organisms or earthworms.

Additionally, the impact on microbial activity in soil such as an impact on nitrogen and carbon mineralization processes after the use of the product has also to be assessed although no experimental data are required where it can be justified that a risk assessment can be performed with the available information.

10.5 Recommended Guidelines

Annex IIB of Directive 91/414/EC specifies that the information required shall be generated using available test guidelines accepted by the competent authority, referring to the US EPA guidelines for testing microbial pesticides (EPA 1996). If appropriate test guidelines are described in Annex IIA, they should be adapted in such a way that they are appropriate for micro-organisms.

11 Ecotoxicity assessment of GM plant proteins – the regulatory context

11.1 Risk assessment requirements in the United States

In the United States the regulation of genetically modified plants (GMPs) is covered by the regulation of biopesticides. Biopesticides include biochemical pesticides, microbial pesticides and plant-incorporated protectants (PIPs). In the case of pesticidal substances produced in plants (PIPs), the protein and its genetic material but not the plant itself are regulated by the US Environmental Protection Agency (US EPA). Consequently the risk assessment requirements of GMPs are similar to those required for plant protection products when assessing plant-incorporated protectants.

Guidelines for risk assessment procedures of GMPs in the US have focussed on the general guidelines for risk assessment as provided by the US Environmental Protection Agency (EPA 1998). The ecological risk assessment of GMPs often follows a tiered or step-wise approach. This tiered testing approach starts with testing of transgene products on selected non-target species under laboratory conditions and progressing to more realistic conditions using the whole GM plants in field trials. The laboratory studies should aim at evaluating the sensitivity of a given species and the response of a non-target organism to a range of concentrations of the transgene product in combination with the estimation of its likely exposure to the product in the field (O'CALLAGHAN et al. 2005). Those non-target organisms for which toxicity is demonstrated are subject to a more detailed analysis and higher tier tests. If the risk is considered acceptable or negligible the risk assessment stops at this point. The major goal of this approach is to identify toxic proteins at an early stage and concentrate on those substances that pose risks thus preventing excessive and expensive testing. This kind of toxicological testing has its origin in chemical pesticide testing, establishing worst case conditions for the non-target organism, i.e. direct exposition to the toxin (or protein in case of GMPs) at high concentrations. Such toxicological tests are standardised, repeatable, interpretable and relatively simple in design (ROMEIS et al. 2006b).

With respect to environmental issues the testing requirements for biopesticides include an assessment of the toxicity of the product to birds, fish, earthworms and representative insects such as bees, ladybird beetles and lacewings, on insects related to target insect pests as well as mammalian toxicity. Also the length of time required for new proteins to degrade in the environment is a necessary data requirement when a biopesticide is assessed.

The assessments of **Bt** corn and **Bt** cotton plant-incorporated protectants are contained in the Biopesticides Registration Action Documents on **Bt** plant incorporated protectants (EPA 2001). Data requirements are presented for the Cry1Ab and Cry1F protein including toxicological tests with honey bee larvae and adults, parasitic wasps, ladybird beetles, green lacewings, springtails, earthworms, the Monarch butterfly larvae and daphnids. **Bt** corn in the diet was tested with birds (bobwhite quail) and fish (catfish).

Soil persistence is usually assessed by the determination of degradation times of the purified proteins.

11.2 Risk assessment requirements in the European Union

In the European Union the principles for the risk assessment of GMPs are laid down in Annex II of Directive 2001/18/EC (EUROPEAN COMMISSION 2001b) and the Guidance Notes for Annex II (EUROPEAN COMMISSION 2002). The EU risk assessment approach is considered broader than the US approach as it includes immediate and delayed effects as well as long-term effects resulting from direct and indirect interactions of a GMP with its environment including competitors, herbivores, symbionts, parasites and pathogens. The Guidance Notes on Annex II specify that the evaluation of potential adverse effects should be based on scientific and technical data and on common methodology for the identification, gathering and interpretation of the relevant data and addresses the uncertainty of the scientific methods (EUROPEAN COMMISSION 2002, point 3). The Guidance Notes also emphasize the usefulness of the step-by-step approach, beginning with experiments in the contained use system through the deliberate release up to the placing on the market.

For GMPs placed on the market according to the Regulation (EC) 1829/2003, the Guidance Document published by the European Food Safety Authority (EFSA 2006) emphasizes the possibility to conduct an environmental risk assessment in a tiered manner according to WILKINSON et al. (2003), starting with hazard identification at tier 1 (controlled lab or growth room studies), continuing with tier 2 (trophic layer effects, controlled lab or glasshouse conditions) and finishing with tier 3 (field studies). The EFSA further refers to the use of the tiered approach for the assessment of interactions of the GMP with non-target organisms (EFSA 2006, see Chapter 9.5).

The Guidance Notes supplementing Annex II to Directive 2001/18/EC lay down the objective, elements, general principles and methodology for the environmental risk assessment approach of GMPs (EUROPEAN COMMISSION 2002). The following main steps should be addressed in the environmental risk assessment:

Step 1: Identification of characteristics which may cause adverse effects

Step 2: Evaluation of the potential consequences of each adverse effect if it occurs

Step 3: Evaluation of the likelihood of the occurrence of each identified potential adverse effect

Step 4: Estimation of the risk posed by each identified characteristic of the GMO

Step 5: Application of management strategies for risks

Step 6: Determination of the overall risk of the GMO

The Guidance Notes give examples of potential characteristics and adverse effects of GMOs which may occur and examples of possible consequences of adverse effects if they occur. As a major factor in evaluating the likelihood of an adverse effect (step 3) the characteristics of the environment into which the GMO is intended to be released and the manner of the release is emphasised.

12 Ecotoxicity assessment of proteins in GM plants

12.1 Genetically modified plants containing Cry-proteins

Genetically modified plants (GMPs) have been authorized within the European Union or are pending for authorization under Directive 2001/18/EC or Regulation (EC) 1829/2003. So far, the notifications submitted to the respective regulatory bodies concern maize, soybean, oilseed rape, potato, sugar beet and cotton. The major traits introduced into GMPs currently are herbicide tolerance and insect resistance. So far only few GMPs contain traits aiming at changes in the plant composition such as high-lysine content or amylopectin levels. Notifications covering stacked events, i.e. GMPs which are derived from the traditional crossing of single event GMPs are increasingly submitted. Resistance of GMPs to certain insect pests is still one of the major trait in GMPs. In most cases, genes derived from the common soil bacterium ***Bacillus thuringiensis*** are used as a source for mediating insect resistance to GMPs.

Crops expressing Cry-proteins derived from ***Bacillus thuringiensis*** are maize and cotton (**Bt** maize, **Bt** cotton). Other crops submitted for authorization or already authorized within the European Union such as oilseed rape, potato or sugar beet so far do not contain Cry-proteins. Introduced Cry-proteins comprise Cry1Ab (maize), Cry1Ac (cotton), Cry3Bb1 (maize), Cry34/35Ab1 (maize) and Cry1F (maize). Of the **Bt** maize or **Bt** cotton notifications submitted so far, eleven cover cultivation within their scope of notification (see Table 25). Two notifications of insect resistant crops (maize Bt176, maize 59122xNK603) were recently withdrawn by the applicant.

Table 25: Overview of GMPs expressing Cry-proteins authorised or pending for placing on the market (including cultivation) within the European Union.

GMP	Protein	Notification (scope)	Status
MAIZE			
1507	Cry1F	C/ES/01/01 (import, industrial processing, food and feed use, cultivation)	Pending under Dir 2001/18/EC
Bt11	Cry1Ab	C/F/96/05-10 (application updated 1998 and 2002) (food, feed, industrial processing, cultivation)	Pending under Dir 2001/18/EC
MON810	Cry1Ab	C/F/95/12-02 (seed = cultivation, processing)	Submitted and authorized under Dir 90/220/EEC*
Bt176	Cry1Ab	C/F/94/11-03 (seed = cultivation, processing)	Withdrawn by applicant
59122	Cry34Ab1/Cry35A b1	EFSA/GMO/NL/2005/23 (food, feed, import, processing, cultivation)	Pending under Reg (EC) 1829/2003
MON810 x NK603	Cry1Ab	EFSA/GMO/NL/2005/26 (cultivation only)	Submitted under Reg (EC) 1829/2003
1507 x NK603	Cry1F	EFSA/GMO/UK/2005/17 (food, feed, import, processing, cultivation)	Pending under Reg (EC) 1829/2003
1507 x 59122	Cry1F Cry34Ab1/Cry35A b1	EFSA/GMO/NL/2005/28 (food, feed, import, processing, cultivation)	Pending under Reg (EC) 1829/2003
59122 x NK603	Cry34Ab1/Cry35A b1	EFSA/GMO/UK/2006/29 (food, feed, import processing, cultivation)	Withdrawn by applicant
59122 x 1507 x NK603	Cry1F Cry34Ab1/Cry35A b1	EFSA/GMO/UK/2006/30 (food, feed, import processing, cultivation)	Pending under Reg (EC) 1829/2003
COTTON			
MON 531	Cry1Ac	C/ES/96/02 (incl. cultivation)	Transferred to Reg (EC) 1829/2003

Sources: www.gmo-compass.org/eng/ gmo/db/, <http://gmoinfo.jrc.it/> and EFSAnet, Status: September 2007. *MON810 maize is currently under re-evaluation according to Regulation (EC) 1829/2003.

12.2 Safety assessment of Cry-proteins in GMPs

12.2.1 Cry-proteins in GMPs

The Cry-proteins introduced into GMPs are derived from different bacterial subspecies or strains of *Bacillus thuringiensis*. In most GMPs not the native gene sequences of the source organism is introduced but a modified sequence according to the expression in higher plants and the target species aimed at. Consequently, the resulting protein as expressed in the GMP differs from the native protein to a variable extent.

12.2.1.1 Sequence of Cry-proteins in GMPs

In many cases the introduced sequences in GMPs which are derived from microbial *Bt* strains are composed of a part of the native amino acid sequence, with or without changes in the amino acid sequence (see Table S1 in Confidential Supplement Volume). In the case of the **Cry1Ab** protein as expressed in Bt11 maize the introduced protein constitutes the N-terminal end of the native protein, consisting of 615 amino acids which are unchanged in their sequence compared to the wild type. The sequence of the **Cry1Ab** protein intended to be expressed in MON810 maize is identical to the native sequence. However, in the GMP only part of the native sequence was integrated as the GMP did not express the full length sequence. The **Cry1F** protein expressed in 1507

maize represents 605 amino acids of the native protein sequence. The native amino acid sequence was changed by a single substitution compared to the sequence of the native Cry1F protein. The protein contained in cotton MON531 is a hybrid construct of **Cry1Ac**, derived from a fragment of the native Cry1Ab sequence of 466 amino acids and a fragment of the native Cry1Ac sequence of 711 amino acids. Both sequences differ from the native Cry1Ac sequence by 6 and 1 amino acids, respectively. The total sequence similarity of the resulting protein as expressed in cotton MON531 has a 99.4 % amino acid sequence similarity to the naturally occurring Cry1Ac protein. Genetically modified maize 59122 contains the binary **Cry34Ab1 / Cry35Ab1** proteins. The introduced amino acids are of identical length and sequence compared to the native protein. Several amino acid were substituted in the native **Cry3Bb1** toxin found in microbial **Bt** strains with the aim to increase bioactivity towards the target insects, Southern corn rootworm and Colorado potato beetle. The Cry3Bb1 variant 11098 which itself differs from the wild type by five amino acids was then modified in order to be expressed in transgenic maize MON863. The modified and introduced protein encoded additional two changes relative to the bacterial Cry3Bb1 variant 11098 and differs in total by seven amino acids from the wild type protein. These variants are considered “alleles” of the Cry3Bb1 protein by the notifier as the amino acid sequence difference was less than 5 % of the total wild-type Cry3Bb1 sequence. The bacterial variant 11231 of the Cry3Bb1 protein differs by four amino acids from the wild type Cry3Bb1 protein. The transgenic corn MON853 is based on this variant and differs by one amino acid from the bacterial variant 11231. In the transgenic corn events (MON853 and MON863) amino acid positions are shifted upwards by one amino acid relative to the bacterial proteins due to the introduction of a single amino acid.

12.2.1.2 Size of Cry-proteins in GMPs

Generally studies are submitted by the notifier to demonstrate the equivalency of the bacterially expressed protein and the protein as expressed in the GMP in order to justify the use of these proteins expressed in bacteria in toxicity studies for human or animal health assessment. Microbially expressed proteins are also used to establish the specificity of the protein and in ecotoxicity tests with non-target organisms. The differences between the plant produced proteins and microbially produced proteins is evident from these studies evaluating equivalence of these proteins.

Differences in molecular weight between the protein expressed in GMPs and in bacteria are evident for almost all Cry-proteins (see Table S1 in Confidential Supplement Volume). Besides the expected fragment sizes, frequently smaller fragments (bands) are observed in GMP expressed proteins in Western blots. In some cases the origin of these smaller fragments of the GMP expressed proteins which are not observed in bacterially expressed proteins is considered due to the presence of plant-derived trypsin-like proteases released during the processing of the plant tissues (e.g. the observed “doublet” in Cry1F maize).

12.2.2 Assumptions underlying ecotoxicity assessment of Cry-proteins

In general, notifiers assess effects on non-target organisms through laboratory studies with certain test organisms which are considered to be representatives of the major groups of beneficial organisms, such as predators, parasitoids, pollinators, detritivores and sometimes incidental species. In some notifications the claimed specificity of the Cry-protein is supported by a pest susceptibility study covering a few pest species. Effects of the Cry-protein on biogeochemical processes are usually assessed by a persistence study of the respective proteins in soil under laboratory conditions.

In the notifications of GMPs for cultivation expressing Cry-proteins, the following arguments in order to support the absence of any adverse effect the Cry-proteins may exhibit in the environment are frequently stated.

- Proteins can act only as acute toxins.
- The history of safe use of Cry-proteins as microbial pesticides.
- The natural ubiquity of Cry-proteins in the soil environment.
- The limited persistence of the respective proteins in the soil environment.
- The specificity of the Cry-protein against a certain target organism.

Notifications of stacked events usually refer to the assessments or notifications of the individual Cry-proteins. Potential interactions between different Cry-proteins when expressed in the same plant are usually not addressed by the notifiers. Arguments for the absence of possible interactions between the introduced proteins are:

- The conclusions of the safety assessments for the individual proteins are not changed when their combined expression in the GMO is considered.
- The very narrow spectrum of insecticidal activity of the individual proteins.
- The lack of evidence for synergistic effects in several agronomically important species (frequently citing MACINTOSH et al. 1990).

12.2.3 Specificity assessment of Cry-proteins

This specificity of the introduced Cry-proteins is generally explained by the specific receptors in the gut of the target organism as well as the activation of the toxin by the relevant proteases of the target pest species. Due to this narrow specificity of the biological activity of the Cry-proteins, the absence of any toxicity and consequently the absence of any adverse effect to non-target organisms is assumed.

The specificity of the Cry-proteins introduced into GMPs is usually assessed in studies with selected target pest species. Table S2 in Confidential Supplement Volume) gives an overview of the studies conducted by the notifier, the organisms tested and the proteins used. In most cases only a limited range of insect pest species are selected, in some cases species from different insect orders than the target organism are included (maize MON863, maize 59122, cotton MON531). The microbially expressed proteins and the respective GMP are used as the test substances. In the case of maize 59122 only the microbially expressed proteins were used. The proteins expressed in bacteria are frequently trypsinized to result in a trypsin-resistant protein (e.g. maize Bt11, maize 1507, probably cotton MON531). Non-trypsinized proteins were used in the case of GM maize 59122 (HERMAN 2000). Different microbial proteins (full-length, truncated) were also used in specificity tests for this maize (GAO et al. 2004). In the case of maize MON863 it remains unclear whether the full length microbially expressed protein or whether the trypsin-resistant core protein was used for the specificity tests.

12.2.4 Ecotoxicity assessment of Cry-proteins

Ecotoxicity assessment of Cry-proteins is usually composed of an assessment of effects of the introduced proteins on selected non-target species (effects assessment) and an assessment of the degradation of the protein in the soil environment. Exposure assessment is not constantly included in the notifications.

12.2.4.1 Exposure assessment

An assessment of the exposure of non-target organisms to the introduced protein in the respective GMP is not constantly contained in the notifications of GM plants for cultivation (Table S3 in the Supplement Volume). Only three out of eleven notifications

for the cultivation of a GMP contained a separate exposure assessment. In those notifications which contained an exposure assessment, it is generally based on the expression levels of the respective GMP. However, in two cases expression levels from GMP cultivation trials outside the EU were used (maize 59122: Chile; maize MON863: Argentina).

Exposure assessment was conducted separately for plant feeding organisms and soil dwelling organisms in the case of maize MON863. In the notification of maize 59122 taxa from several trophic levels (phytophages, higher trophic levels, pollinators, detritivores, incidentals, aquatic species) were considered. In the case of maize 1507 only lepidopteran species were included in the exposure assessment.

Notifications of stacked events usually do not contain a separate exposure assessment. In these notifications it is referred to the safety of the introduced proteins as either shown in the single event notifications or acknowledged by opinions of regulatory authorities (e.g. EFSA opinions of single events).

12.2.4.2 Effects assessment

The ecotoxicity tests provided by the notifiers in order to prove the safety of the respective Cry-toxin of a GM crop to non-target organisms consist in most cases of company internal reports (Table S4 in Supplement Volume). In some cases toxicological studies are cited by the notifier but not attached to the notification (e.g. maize MON863, maize 1507, maize MON810, Cotton MON531). In some notifications these studies are attached as confidential information (e.g. maize MON810xNK603) while in others they are not (e.g. maize 1507x59122).

In the case of maize MON863 an appendix is attached to the notification citing the toxicological studies assessing the safety of the Cry3Bb1 and the Cry1Ab toxins. However, these studies were not attached to the notification. Additionally, a study was added (ASTWOOD et al. 2001) again citing the results of some of these toxicological studies. In the case of the binary Cry-toxins Cry34Ab1/Cry35Ab1 of the single event notification of maize 59122 the toxicological studies were summarized in a company internal study (POLETIKA 2003) but not attached to the notification (except one study: HERMAN 2000). However, these studies were later attached to the notification of the stacked event 1507x59122.

For the toxicity assessment of a specific Cry-toxin, the same studies are usually provided by the notifiers. The studies assessing effects of the Cry1Ab protein on honey bees (MAGGI & SIMS 1994a, 1994b) were provided in all maize notifications dealing with a GMP containing the Cry1Ab toxin (maize Bt11, maize MON810 and maize MON810xNK603). Similarly, studies for effects of Cry1Ab on green lacewings (HOXTER & LYNN 1992a), parasitic hymenoptera (HOXTER & LYNN 1992b) and ladybird beetles (HOXTER & LYNN 1992c) were all provided in the above mentioned notifications.

Generally, the safety assessments of the Cry-toxins contained in stacked events refer to the single event notifications and assessments. Thus, toxicological studies of Cry-toxins used in stacked event notifications are usually the same as for single event notifications. In the case of GM maize 1507x59122 the notifier attached all studies for the assessment of the Cry1F toxin (except BYSTRAK 2000) which were already mentioned in the notification of maize 1507. All studies for the toxicological assessment of the binary toxin Cry34Ab1/Cry35Ab1 (except VINALL 2005) cited in the notification were already cited in the maize 59122 notification, but not attached (see above).

Methods employed and parameters assessed

Non-target toxicity tests are designed to expose certain non-target species to concentrations of the respective Cry-proteins that are comparable or higher than those expressed in the GM plant. These tests are either “dietary toxicity/effects studies” (e.g.

ladybird beetles, green lacewings, rainbow trout, honey bees, hymenoptera, birds) or acute toxicity studies (e.g. earthworms, daphnids). Chronic toxicity is assessed only in studies with *Collembola* (see Table 26 and Table S4 in Supplement Volume).

Standardized protocols for toxicity tests are generally not followed. Test protocols according to US EPA microbial pesticide test guidelines were followed in one of four studies with ladybird beetles, in two of three tests using green lacewings, one of four tests with honey bees and one of three tests with parasitic hymenoptera. OECD guidelines for testing of chemicals were followed for all four acute toxicity tests with earthworms, as well as all three tests with *Collembola* (reproductive toxicity), one acute toxicity study with daphnids and one acute toxicity test each with fish and birds. The only ground beetle test submitted followed the methodology for testing effects of plant protection products in the laboratory published by the IOBC/WPRS.

A test substance, the purified, microbially produced Cry-protein, in some cases pollen or both (e.g. honey bees), is added to a laboratory diet or a solution and offered to the respective test organism as the sole food source (see Table 26). In the case of the lacewing larvae the test substance is usually applied with moth eggs. In the test with the ground beetle the test substance was injected into a prey organism (fly pupae) provided to the ground beetle larvae.

Test substance concentrations vary between tests using the same test organisms. The choice of test substance concentrations is not always justified by the authors. In a few cases the test concentrations are related to the environmental concentration expected for the respective test organism, e.g. 10 times the expected concentration of the protein in GMP pollen for ladybird beetle tests.

The length of the testing period varies considerably between tests applied to the same organisms. For example, in the case of tests with ladybird beetles the testing period ranges from 7 to 29 days. In some of these tests the test was stopped due to the mortality in the control groups (Table S5 in Confidential Supplement Volume).

Control groups are generally included in the tests. As negative control substances the carrier without the test substance is frequently used (e.g. yeast only, *Collembola*). Where the GM plant is used as the test substance, a non-GM plant was included as control (e.g. *Collembola*). The heat inactivated microbial protein is also used as a negative control, e.g. in tests with ladybird beetles. Positive controls are not constantly included in the toxicological tests. Positive controls are generally absent from tests with ladybird beetles, lacewings, parasitic hymenoptera, birds and fish but are included in tests with earthworms, *Collembola*, daphnids or ground beetles. Only one honey bee test following the US EPA microbial test guideline used a positive control. In many tests the performance of the control groups is unsatisfactory. Tests are stopped due to high mortalities (higher than 20 %) in the control groups or mortalities are higher in the negative control groups than in test groups, especially in tests with ladybird beetles, green lacewing larvae and parasitic hymenoptera (see Table S5 in Confidential Supplement Volume).

Sample sizes per treatment and number of replications vary considerably between tests with specific non-target organisms, only in earthworm and *Collembola* tests sample size and replication was constant (see Table 26).

Assesment of Toxic and Ecotoxic Properties of Novel Proteins in GMOs

Table 26. Overview of testing methods applied and test organisms commonly used for the assessment of Cry-toxins in notifications for cultivation of GMPs.

Test organism	No	Test substance	Test substance applied with	Replication	Individuals per Replicate	Length of study	Test guidelines (x)
Daphnids	1	Microbial proteins	Water	3	10	48 hrs	OECD No. 202: Daphnia acute immobilization test
Honey bees	3 larvae 1 adult	GMP pollen; purified protein	Sucrose solution, distilled water, honey-water mixture	Larvae: 3-4 Adult: 3	Larvae: 20 or 50 Adult: 40	Larvae: 6-26 d Adults: 9 d	EPA microbial pesticide test guidelines Group D (1)
Green lacewing larvae	3	Microbial protein	Moth eggs	4 or 30	1 or 10	7 - 13 d	Based on OPPTS No. 885.4340 (2)
Parasitic wasps	3	Microbial protein	Honey:water solution, commercial honey, sugar water	2, 3 or 4	25	11 - 30 d	OPPTS No. 885.4340 (1)
Ladybird beetles	4	Purified protein, Pollen expressing proteins	Honey-water solution, commercial honey, sugar water; artificial diet	Larvae: 30 or 40 Adult: 2, 3 or 6	Larvae: 1 Adult: 25	7 – 29 d (until emergence)	OPPTS No. 885.4340 (1)
Ground beetles	1	Protein mixture	Injected into prey	40	1	Until pupation	Heimbach (1998)
Collembola	3	Protein extracted from GMP leaves (lyophilized corn leaf tissue) Microbial protein	Yeast, artificial soil	4	10	28 d	OECD: Reproductive toxicity for Collembola (3)
Earthworms	4	Microbial protein in powder diluted in water	Artificial soil substrate	4	10	14 d	OECD, No. 207: Acute toxicity for earthworms (4)
Birds	1	Grain containing Cry-protein	Basal diet	4	5	8 d	OECD, No. 205: Avian dietary toxicity
Fish	2	Grain containing Cry-protein, bacterially produced protein	Commercial fish diet	3 or 5	10 or 20	8 w, 8 d	OECD No. 203: Fish acute toxicity test (1)

“Tox test applied” refers to the designation of the test by the notifier. hrs = hours, d = days, w = weeks. No = number of studies assessed. (x) = number in brackets indicates the number of studies following or based on the test guideline.

Table 27 gives an overview of the observed parameters in the toxicological tests. The parameters chosen aim at assessing acute toxicity, such as mortality. Studies assessing chronic effects or reproductive toxicity are rarely submitted (e.g. Collembola). Multi-generational studies are generally missing. Several parameters which are assessed are not described in detail. For example, the parameters “signs of toxicity” or “abnormal behaviour” are rarely defined. It remains unclear which effects are assessed under these terms. In case sublethal effects are observed, they also lack detailed description. Only in one test assessing sublethal parameters in earthworms, these were classified as “elongated or lethargic” although a detailed definition of these parameters is also lacking.

Table 27. Toxicological parameters assessed in the assessment of Cry-toxins on commonly used test organisms in notifications for cultivation of GMPs.

Test organism	Observed parameters
Daphnids	Immobility
Honey bees	Mortality, signs of toxicity, adult emergence
Green lacewing larvae	Mortality, signs of toxicity, pupation, abnormal behaviour
Parasitic wasps	Mortality, signs of toxicity, abnormal behaviour
Ladybird beetles	Mortality, signs of toxicity, abnormal behaviour, development, weight at emergence
Ground beetles	Pre-imaginal mortality, body weight of newly-emerged adult beetles
Collembola	Mortality, sublethal effects, reproduction (no of offspring)
Earthworms	Mortality, signs of toxicity, body weight, burrowing time, sublethal effects
Birds	Mortality, signs of toxicity, abnormal behaviour, body weight, feed consumption
Fish	Weight gain, feed consumption/conversion, mortality, behaviour, sublethal effects

Not all parameters listed were included in each study.

Species used

Generally, a similar set of test species is chosen by the notifiers. Species usually tested are honey bees, green lacewing larvae (*Chrysoperla carnea*), parasitic wasps (*Brachymeria intermedia*, *Nasonia vitripennis*), ladybird beetles (*Hippodamia convergens*, *Coleomegilla maculata*), Collembola (*Folsomia candida*), earthworms (*Eisenia fetida*) and daphnids (*Daphnia magna*).

In two notifications non-target lepidopteran species were included in the toxicological assessments (maize 1507 and maize 59122), using the monarch butterfly (*Danaus plexippus*). In one notification a study using ground beetles (*Poecilus cupreus*) was included (maize 1507x59122).

In a few notifications also vertebrates are tested such as birds (*Colinus virginianus*) and fish (*Oncorhynchus mykiss*, *Ictalurus punctatus*).

For some taxa only larval species are tested (e.g. green lacewings) while for other taxa no larval stages are included in the tests (e.g. earthworms). The age of larvae is frequently not indicated or ranges from newly hatched to few days old (e.g. green lacewings). Ladybird beetles were either tested as adults or as neonate larvae. Honey bees were mainly tested as larvae but also as adults. Either adult parasitic hymenoptera were used in the tests or the age of the individuals was not indicated.

12.2.4.3 Degradation assessment of Cry-proteins in soil

Notifiers of GMPs usually assess the degradation of the introduced protein in soil (Table S6 in Supplement Volume). The studies are either submitted as company internal studies (maize Bt11, maize 1507, maize 59122, maize MON863) or the notifier refers to studies published in peer-reviewed journals. In some notifications protein degradation studies are not attached to the notification (maize MON863) and only the results are presented. The notification of cotton MON531, maize MON810 and the stacked events do not contain any studies conducted to assess the degradation of the introduced Cry-proteins in soil.

Studies conducted by the notifiers usually comprise laboratory studies in which either GM plant derived tissue or more frequently the purified proteins are added to soil and incubated in the laboratory at room temperature for a short time (maximum 28 days). In certain intervals samples are taken and the insecticidal activity is determined by a bioassay with a susceptible insect larvae. As a result the DT_{50} and the DT_{90} (time until 50 % or 90 % of the concentration of the toxin is not detectable) is presented.

13 Critical appraisal of assumptions underlying ecotoxicity assessment of GM plant proteins

In the notifications of GMPs several assumptions are made for the toxicity assessment of proteins expressed in GMPs. In this chapter some of these assumptions are critically analysed and their shortcomings discussed.

13.1 Proteins are only known to act as acute toxins

13.1.1 Delineation of toxic effects of proteins

Toxic, anti-digestive or anti-nutritive effects of phytochemicals can be distinguished (DUFFEY & STOUT 1996). “Toxic” signifies the detrimental effect upon the living tissues and/or internal processes of an insect (e.g. interaction with the luminal surface of midgut cells or passing through the gut wall of a chemical). Other external processes are termed differently depending on whether the chemical limits the rate of enzymatic conversion of food (anti-digestive) or if it limits utilization by altering physical availability or chemical identity (anti-nutritive).

The denomination of a protein being a “toxin” without considering the biological and chemical context is considered highly difficult. Without any definition of the specific context the differentiation of a toxin, a digestibility reducer, an antinutrient or a nutrient is not considered as useful as all these effects depend on the specific context (DUFFEY & STOUT). Therefore effects of toxicity, digestibility reduction or nutrification are not considered inherent properties of a chemical or the result of a mechanism for each effect (e.g. alkylation or binding to a substrate) but all of them may arise from the same mechanism. Due to the ability to undergo various intermolecular interactions no individual substance has unique properties which makes it possible to classify it as toxin, antinutrient and/or digestibility reducer (DUFFEY & STOUT 1996).

For example, proteinase inhibitors have been shown to have both, toxic and anti-nutritive, but not anti-digestive effects upon noctuid larvae as the effect is dependent upon the chemical (type of proteinase inhibitor), physical (pH) and biological (insect species) context (DUFFEY & STOUT 1996).

13.1.2 Effects of proteins in GMPs other than acute effects

Generally, the evaluation of the insecticidal activity of Cry-proteins is derived from tests with a few agronomically important insect species usually through bioassays evaluating the acute toxicity by establishing the LC_{50} value (see e.g. HÖFTE & WHITELEY 1989, MACINTOSH et al. 1990) or growth inhibition in cases where no mortality occurred (HERMAN et al. 2002b). The definition of a susceptible insect is generally related to agronomic terms, i.e. an insect that is quickly killed with one or a few applications requiring an acute and lethal effect (HILBECK & SCHMIDT 2006). Consequently, information on sub-lethal effects of Cry-proteins were rare until the first studies using GMPs discovered such kind of effects (see following Chapter).

13.1.2.1 Bacterial proteins (Cry-proteins)

Sublethal effects of delta-endotoxins of *Bacillus thuringiensis* have been described as early as in 1982 by Burges stating that “**sub-lethal doses may act like dysentery in man, with recovery after diarrhoea**”. He also notes that “**successive, sublethal doses may kill larvae or produce pupae, many of which die or produce adults that may die prematurely, often with infertility and reduced egg production**” (BURGES 1982). Also

other evaluations of delta-endotoxin toxicity in the 1980s revealed differences in sensitivity to different species or even insect orders (see e.g. CHILCOTT & ELLAR 1988). Although sublethal effects of Cry-proteins were reported for **Bt** sprays when effects on non-target organisms were investigated, lethal effects were the focus of most reports (GLARE & O'CALLAGHAN 2000 cited in HILBECK & SCHMIDT 2006). FLEXNER et al. (1986) reviewed toxic effects of different commercial microbial formulations of **Bacillus thuringiensis** on beneficial insects. Some of the studies cited by FLEXNER et al. (1986) reported, apart from toxic effects, also sublethal effects such as reduced egg production, reduced host consumption, reduced parasitism, reduced adult longevity, increased parasitism or parasitoid development and skewed sex ratios of several non-target beneficial organisms.

With the rise of Cry-proteins incorporated into GMPs, studies focussing on non-target organisms and other effects than acute toxicity appeared (HILBECK et al. 1998b, HILBECK et al. 1999, LOSEY et al. 1999) although studies dealing with effects of GMPs on non-target invertebrates still focus on lethal effects (HILBECK & SCHMIDT 2006).

The following sublethal effects of Cry-proteins were reported in the literature which were evaluated by feeding the toxin either via bioassays, via the GMP or via a GM-fed prey species:

- Reduced consumption, slower growth and reduced weight of non-target lepidopteran species (LOSEY et al. 1999, HILBECK et al. 1999, FELKE et al. 2002, DIVELY et al. 2004, ANDERSON et al. 2004, 2005).
- Increased developmental time of parasitoids (Meissle et al. 2003), green lacewing larvae (HILBECK et al. 1998b) or non-target Lepidoptera (DIVELY et al. 2004, ANDERSON et al. 2004, DUTTON et al. 2005, VOJTECH et al. 2005).
- Incomplete development and lower emergence of parasitoids (SCHULER et al. 2004, PRÜTZ & DETTNER 2004, MEISSLE et al. 2003) or reduced pupation and adult emergence of coccinellids (SHARMA et al. 2006).
- Shorter live span of non-target lepidoptera (ANDERSON et al. 2004, 2005, DUTTON et al. 2005, VOJTECH et al. 2005).
- Lower survival of predacious carabids (MEISSLE et al. 2005) or coccinellids (SCHMIDT et al. 2004).
- Reduced fecundity or weight of parasitoids (PRÜTZ & DETTNER 2004, PRÜTZ et al. 2004).
- Reduced cocoon weights of parasitoids (VOJTECH et al. 2005).

LÖVEI & ARPAIA (2005) reviewed 26 published papers evaluating 18 species of predators in laboratory tests (35 experiments) with GMPs. For predator species they found that 30 % of all tests resulted in significantly, 11 % in non significantly negative effects. Among the significantly negative effects most were reported for the parameters egg viability, behaviour, longevity, enzyme activity, reproduction, prey consumption, development time and body mass or size (22–80 % of the cases) while survival and mortality accounted for only 15.5 % of the “significantly negative” cases (LÖVEI & ARPAIA 2005). Similarly, the analysis of 128 tests using parasitoids showed that the majority of the parameters were significantly or non-significantly negative. Most frequently parasitoid longevity was affected (59 % of cases) followed by mortality, emergence and development time (50 %), fecundity (48 %), parasitism (35 %), body mass or size and behavioural differences (33 and 27 %, respectively). These results show that a range of sublethal effects were observed for organisms related to GMPs and that acute toxic effects are less frequent than generally presumed.

13.1.2.2 Plant proteins (Lectins)

Plant lectins such as the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) can exhibit both antifeedant and toxic effects (POWELL et al. 1995, POWELL et al. 1998 cited in POWELL 2001). GNA has shown to be a potent feed inhibitor and caused mortality in a range of insect pests such as aphids and leafhoppers. The snowdrop lectin expressed in genetically modified plants was shown to reduce fecundity and retard development of aphids (DOWN et al. 1996) and exhibited a range of sublethal effects on ladybirds when feeding on GM potato raised aphids (BIRCH et al. 1999). The latter study showed that no acute toxicity effects on the ladybirds occurred but fecundity, egg viability and longevity decreased significantly although the adverse effects on reproduction of the ladybirds were reversible. Another study showed effects of the GNA on a parasitoid developing in GNA-fed aphids (COUTY et al. 2001). The observed effects on the female parasitoids (smaller size, reduced longevity and fecundity) were host-mediated via reduced aphid size but a direct effect of the lectin on the parasitoid was also confirmed. The authors thus suggested an antifeedant effect of the GNA on the parasitoid larvae (COUTY et al. 2001).

13.2 Proteins act specifically against target organisms

13.2.1 Hypothesis of a lack of exposure of non-target organisms

The specific exposure of different non-target organisms or the existence of different exposure pathways are usually not considered in notifications for placing on the market for cultivation of a GMP within the EU. In few notifications an exposure analysis is conducted. If such an analysis is included in the notification it is mostly based on the expression levels of the protein in the GMP and therefore only a direct exposure via the GMP is considered. Other exposure pathways are generally not taken into consideration by the notifiers (see Section 12.2.4).

13.2.2 Evidence for the existence of different exposure pathways

Organisms which are present in agro-ecosystems can be exposed to Cry-proteins via different pathways. The expression levels of Cry-proteins generally differ depending on the location but also at a specific location between plant organs. Expression levels have been shown to be different depending on the age of the plant (DUTTON et al. 2004), age of the leaves (DUTTON et al. 2005) and the leaf section as well as position of the leaf on the plant (ABEL & ADAMCZYK 2004). Additionally, certain GM plants release Cry-proteins via their roots into the soil thus contributing to toxin residues in soil, either bound to soil particles or free (SAXENA et al. 2002, 2004).

After harvest, plant residues such as leaves and particularly roots remaining on the field constitute a considerable reservoir for Cry-proteins (BAUMGARTE & TEBBE 2005) being available for organisms which degrade plant material above and below ground. Additionally, soil organisms such as isopods ingest and excrete these toxins thus making them available to other non-target organisms (WANDELER et al. 2002).

Apart from GM plants and their post-harvest residues, it has been shown that non-target organisms such as herbivores but also predatory species contain Cry-toxins. HARWOOD et al. (2005) have shown that a range of non-target herbivores and natural enemies of several orders contained the Cry1Ab toxin. The presence of these toxins in non-susceptible herbivores such as spider mites, which were shown to contain toxin levels as high as in GM maize (DUTTON et al. 2002) or considerably higher (OBRIST et al. 2005), may present a pathway to higher trophic level species under field conditions.

However, it remains unclear to what extent these toxins are degraded and under which conditions these toxins maintain their insecticidal activity after gut passage of a non-susceptible insect when being available to higher trophic levels.

The presence of Cry-proteins in headwater agricultural streams in corn cropping areas due to the presence of corn pollen or unharvested crop byproducts were recently described by ROSI-MARSHALL et al. (2007).

13.2.3 Hypothesis of the specificity of proteins in GMPs

Generally, the specificity of Cry-proteins is considered to be due to the presence of toxin-specific, high-affinity binding sites in the epithelium of the target insect's gut and to the conditions in the gut affecting the solubilization and/or processing efficiency of the protoxin (HÖFTE & WHITELEY 1989). The respective toxin is considered to be solubilized under the appropriate pH conditions in the target insect's gut and then subjected to proteolysis by the respective gut proteases resulting in an activated toxin that specifically binds to target receptors present only in the target insect's epithelial midgut cells.

Cry-proteins as expressed in GMPs differ in most cases from the native Cry-proteins. The safety of an introduced Cry-protein and consequently of the genetically modified plant to non-target organisms is usually considered due to the specificity of the biological and biochemical activities of the introduced Cry-proteins for the target organism (see also Section 12.2.3).

13.2.4 Factors affecting toxicity and specificity of Cry-proteins

All steps which are necessary for the mode of action of the *Bt* delta-endotoxins have been shown to contribute to the toxin's specificity: solubilization, activation by insect gut proteases, toxin binding and pore formation. Consequently, several factors contribute to or modulate the specificity of Cry-toxins when ingested by an insect.

13.2.4.1 Cry-toxin characteristics

Cry-proteins expressed in GMPs are usually not identical to the native Cry-proteins. Changes in the amino acid length or amino acid sequence are frequently made when Cry proteins are expressed in GMPs (see also Section 12.2.1). In cases where not the full length protein is expressed, the activated protein is considered not dependent on proteolytic processing to be biologically active in the target insect. In other GMPs the full length protein is expressed, making proteolytic processing a prerequisite for biological activity in the target pest species. Also small differences in the amino acid sequence as expressed in GMPs compared to the wild type can also result in differences in the protein size, its structure and in some cases also the biological function. It is recognized that allele differences can result in differences in specific biological activity against the target insect. In the case of the Cry3Bb1 protein, allele variants vary in their biological activity by 2 to 8-fold against the target insect, the Southern corn rootworm (ASTWOOD et al. 2001). There is evidence from alpha-amylase inhibitors that small sequence differences can result in differences in the effectiveness of this protein against a certain target species (FRANCO et al. 2002). However, generally changes in the amino acid sequence are not considered sufficient by the notifiers to create differences in the biological activity affecting insects in different insect orders.

13.2.4.2 Conditions in the insect's midgut

Solubilization and activation of ingested protoxins in the insect's midgut is a crucial step in the mechanism of action of *Bt* endotoxins. Favorable conditions in the insect's gut are necessary for the activation of these proteins as some are only active after dissolution

and activation (see references in ZALUNIN et al. 2004). Differences in solubilization and activation contribute to differences in the toxicity of these proteins (SCHNEPF et al. 1998, KOZIEL et al. 1993). The activity spectrum of the endotoxins might even be broadened by the proper activation (KOZIEL et al. 1993).

Both, the pH and the range of digestive enzymes in the insect's midgut influence the solubilization and activation of the protoxin. However, the solubilization step is necessary for crystal dissolving which is not relevant for Cry-proteins expressed in GMPs.

Suitable protease enzymes in the insect's gut are necessary to release the delta-endotoxin from the protoxin which is the biologically active form. Although in most GMPs the activated toxin is expressed, in some the full length protein is expressed which needs to undergo a proteolytic activation step. It has been suggested that the major proteases of lepidopteran insects are trypsin or chymotrypsin-like (SCHNEPF et al. 1998). Coleoptera seem to have a wider range of dominant gut proteinases (see SCHULER et al. 1998 for references). There is evidence that the protoxin from *Bt* var. *colmeri* HD-847 can be activated into either a dipteran or a lepidopteran toxin depending on the gut proteases (HAIDER et al. 1986 cited in DEML et al. 1999). Activation of Cry-proteins by the respective digestive proteolytic enzymes also differs between the larger (130-140 kDa) and the smaller (70–75 kDa) Cry-proteins (ZALUNIN et al. 2004).

13.2.4.3 Further proteolytic degradation

After proteolytic degradation the resulting active toxin is considered resistant to further degradation although it has been shown that some toxins are subjected to further proteolysis. The spatial structure of the respective toxin determines the process of activation in the gut and consequently its toxicity (ZALUNIN et al. 2004). Toxin proteolysis depends on several factors, such as the enzyme composition, the presence of endogenous and exogenous protease inhibitors, temperature and the rate of gut emptying.

Different delta-endotoxins may also differ in the necessity to be proteolytically activated. It has been shown that the Cry1Ac toxin (135 kDa) breaks down when treated with digestive fluid of lepidopteran larvae into a smaller activated toxin (55-68 kDa) while with the Cry3A toxin (67 kDa) no smaller toxin was found when exposed to digestive fluids of two coleopteran larvae (SLANEY et al. 1992). The authors therefore suggested differences between the proteolytic activation as a possible cause for the observed differential toxicities of the toxin to the two insect species tested. The Cry3A toxin, being insoluble under pH conditions of their target insect's midgut has been shown to retain full activity when proteolytically processed beforehand (CAROLL et al. 1997).

Activation of protoxins may result in toxins with differing specificities (HAIDER et al. 1986 cited in HÖFTE & WHITELEY 1989). The treatment of the full length (130 kDa) protein with trypsin yielded a 55 kDa fragment toxic to lepidopteran cells but not to dipteran cells. However, when the activated toxin was incubated in dipteran gut conditions the resulting 53 kDa fragment was only toxic to dipteran cells. This is also supported by the results of CHILCOTT & ELLAR (1988) who found different toxicities of different Cry-protein fragments of *Bt* subsp. *israelensis* against dipteran and lepidopteran cell lines.

Additional proteolysis has been shown to change significantly the biological activity of a protein. Sequential two-step proteolysis of the P2 protein component of a *Bt* subsp. *kurstaki* HD-1 strain with a lepidopteran gut extract and subsequently with chymotrypsin resulted in a larvicidal and cytolytic protein compared to a treatment with gut protease alone which resulted only in *in vivo* toxicity but no cytotoxicity (NICHOLLS et al. 1989). Similar observations were reported from the *Bt* subsp. *aizawai* IC-1 protoxin which was shown to have a differential specificity correlated with sequential proteolysis

by gut proteases from two different insect orders or by a combination of gut proteases and trypsin (HAIDER et al. 1986 cited in NICHOLLS et al. 1989).

13.2.4.4 Toxin binding to the insect's midgut

Receptor binding of the Cry-toxin to the insect midgut epithelium is considered an important determinant of specificity. The specific receptor sites in the membrane of the insect midgut have been considered one of the keys for the specificity of the *Bacillus thuringiensis* delta-endotoxins. Also the amino acid sequence of the receptor binding domain of the delta-endotoxin is considered to be responsible for host specificity (HÖFTE & WHITELEY 1989).

The observation that certain delta-endotoxins are active against multiple insects (even in different orders) lead already early to the suggestion that either multiple binding sites are present or a particular binding site is present on a number of different receptor molecules whose sequence is conserved among different insects or even insect orders (KOZIEL et al. 1993). However, it has been suggested by other authors that binding of the toxin is not sufficient in order to exert insecticidal activities. Lepidopteran-active Cry-proteins ineffective against a non-susceptible organism (*Lygus hesperus*; Heteroptera) were shown to bind to their gut tissues although the proteins were also detected in the faeces (BRANDT et al. 2004). Reports on adverse effects of *Bt* toxins on non-target organisms such as the green lacewing larvae (HILBECK et al. 1998b) and subsequent studies suggesting that specific receptors for Cry1Ab or Cry1Ac are absent in this organism (RODRIGO-SIMON et al. 2006) leave it unclear whether binding of the toxin as such is conditional for the toxic mode of action of a *Bt* protein.

The exact binding mechanism of the Cry-toxins seems to be still unclear. A sequential role of two receptors, a cadherin-like protein and an aminopeptidase-N (APN) in toxin membrane insertion, pore formation and toxicity of Cry1A toxins has been recently suggested (GOMEZ et al. 2006). Binding of the monomeric Cry1Ab-toxin to the cadherin-like protein facilitates the formation of a pre-pore oligomeric structure which has a higher affinity to the APN thus inserting into the membrane.

Recently, 17 aminopeptidase-N receptors have been shown to bind to Cry-toxins and and for some of those receptors glycosylation is important in the binding process (PIGOTT & ELLAR 2007). In lepidopteran larvae 38 members of the APNs in five different classes have been described. Several different APNs are simultaneously expressed in the larval gut but only two APNs have been shown to mediate toxin susceptibility. There is also evidence that also the cadherin-like proteins are major determinants of Cry1A specificity in lepidopterans (PIGOTT & ELLAR 2007).

The following steps are required for the binding of Cry toxins in Lepidoptera (BRAVO et al. 2007): binding of the monomeric toxin to the first receptor including a conformational change of the toxin, oligomer formation, binding of the oligomeric toxin to the second receptor and conformational change and finally insertion into lipid rafts and pore formation. As a necessary step in the mechanism of action of Cry-toxins the formation of a soluble oligomeric structure before membrane insertion has been suggested by the authors. With respect to receptors BRAVO et al. (2007) considered both cadherin and APN receptors as necessary for full Cry1A toxicity.

Also a mechanism excluding the binding step was recently proposed, based on the interaction of the monomeric Cry1Ab toxin with the cadherin-like protein activating a signalling pathway that leads to apoptosis and not to pore formation thus excluding the insertion of an oligomeric toxin into the membrane (ZHANG et al. 2006). Recently, the importance of the oligomerization and the toxin pore formation for the toxicity of Cry1Ab toxin to lepidopteran insect larvae was again emphasized (JIMENEZ-JUAREZ et al. 2007).

13.2.4.5 Other factors influencing susceptibility

Lysis of the epithelial cells and disruption of the osmotic balance in the insect midgut have been considered the final steps in the mechanism of action of Cry-proteins. The exact cause of death of insects ingesting **Bt** toxins was argued to be due to several factors, such as insect starvation (HÖFTE & WHITELEY 1989), leakage of gut contents into the haemocoel (DEAN et al. 1996 cited in HILBECK & SCHMIDT 2006) or septicaemia by **Bt** spores (DE MAAGD et al. 2001 and BRAVO et al. 2005 cited in BRAVO et al. 2007). A range of factors might therefore influence the effect exhibited by a certain Cry-protein and possibly modulate its toxicity when ingested by an insect.

Host plant composition / nutrient availability

Both, an insect's diet and the composition of the host plant can influence the toxicity of Cry-toxins. Herbivores may be influenced by plant secondary compounds. When **Bt** was incubated with the phytochemicals chlorogenic acid and polyphenol oxidase enhanced solubilization and proteolysis of crystal proteins were observed (LUDLUM et al. 1991). Nicotine concentrations were shown to influence the susceptibility of the tobacco budworm larvae to **Bt** (KRISCHIK et al. 1988 cited in GROTT & DICKE 2002). Purified tannins from cotton fed to the cotton pest species *Heliothis virescens* reduced feeding therefore antagonizing the adverse effects caused by **Bt** (NAVON et al. 1993).

The action of a certain class of compounds (e.g. proteinase inhibitors, proteins, phenolics etc.) upon an insect is rarely independent of other co-ingested chemicals. Its biological activity has often been shown to be context-dependent. The impact of a certain substance depends on its identity and amount, on the identities and amounts of co-occurring chemicals and the physico-chemical environment (DUFFEY & STOUT 1996). No adverse effect of certain proteinase inhibitors was observed in a larval lepidopteran species. The proteinase inhibitors even supplemented the growth and at certain levels of protein acted as nutrients. At a ratio of protein to proteinase inhibitor which reduces growth, the inhibitor is probably digested by the insect's enzymes and the nutritional gain is outweighed by the toxic effects (DUFFEY & STOUT 1996).

A compound's biological activity is not constant but can be altered by the mixture and by the physico-chemical or biological environment (DUFFEY & STOUT 1996). For example, the biological activity of proteinase inhibitors is influenced by dietary protein. A negative impact of a proteinase inhibitor was observed when the protein quantity was suboptimal or supra-optimal. At an optimal protein level the impact was minimal. Also protein quality and other chemicals such as reducing agents and alkylators can play a significant role upon the activity of a proteinase inhibitor because they can deintegrate their disulphide bonds. Similarly, it has been suggested that high pH and redox potential in some insect's digestive fluids can damage disulphide bonds (DUFFEY & STOUT 1996).

Presence of bacteria in the insect's gut

Indications that Cry-proteins influence the midgut bacterial communities in several insect species was already provided by DEML et al. (1999) who found qualitative and quantitative changes of microbial patterns such as a decrease in the total number of colony types or the occurrence of new colony types when insects were fed Cry-proteins. Later the reported antibacterial activity of insecticidal proteins has led to the suggestion that Cry proteins may have a considerable effect on symbiotic micro-organisms of insects (ZALUNIN et al. 2004). Recently, BRODERICK et al. (2006) found that the removal of midgut bacterial community in gypsy moth larvae reduced the larval mortality when fed a commercial formulation of *Bacillus thuringiensis* subsp. *kurstaki* and that midgut bacteria such as *E. coli* or *Enterobacter* spp. are largely responsible for the **Bt** induced mortality in gypsy moth larvae (BRODERICK et al. 2006). The authors therefore suggested that the enteric bacteria are enabled by **Bt** to colonise the hemocoel and cause septicaemia thus contradicting the original assumptions of starvation and septicaemia induced by the **Bt**-formulation itself.

Condition of the organism

The condition of the organism feeding on a GMP may also play a crucial role for the effectiveness of the Cry-toxin against this species. It is recognized that host age of the European corn borer (HUANG et al. 1999) and in general lepidopteran species (e.g. HELLMICH et al. 2001) influences the susceptibility towards the Cry-toxins. Younger instars are generally more susceptible than older. Irrespective of host age also the state of health of the host organism may influence its ability to deal with ingested toxins. It has been shown that infestation of European corn borers with the entomopathogenic microsporidium *Nosema pyrausta* significantly affects susceptibility of host larvae (REARDON et al. 2004). The reduction of the larval weight was significantly reduced and the mortality significantly increased in infected larvae compared to non-infected larvae when fed a Cry1Ab-containing diet.

13.2.4.6 Toxin interactions

Indications of synergistic interactions of Cry-toxins are provided by the observation that the native *Bt* crystals are more insecticidal to a sensitive insect than the individual Cry-toxin compounds. Also potentiating effects between toxins and spores or bacteria have been observed. The combination of several Cry- and Cyt-toxins contained in a single crystal are more insecticidal than the individual toxins or even a mixture (HÖFTE & WHITELEY 1989, SCHNEPF et al. 1998). Assays of different delta-endotoxins, either individually or combined against the dipteran or lepidopteran larvae have revealed synergistic interactions with several combinations of toxins used (see review in SCHNEPF et al. 1998). Synergism of *Bacillus thuringiensis* subsp. *israelensis* crystal proteins against dipteran larvae was shown by CHILCOTT & ELLAR (1988). The most toxic protein against *Aedes aegyptii* larvae was the 65 kDa fragment, but certain protein combinations resulted in higher mosquitocidal activities. The three delta-endotoxins of *Bacillus thuringiensis* subsp. *israelensis* Cyt1, Cry4Aa and Cry11Aa were shown to interact synergistically to kill dipteran larvae when expressed in *E. coli* (KASHDAN et al. 2001). In contrast, mixing of clones expressing different toxins resulted also in synergy although with a lower synergy factor. Also two Cry-proteins (Cry4Aa and Cry11Aa) synergized each other considerably as the larvicidal activity was considerably higher in the clone combining both proteins than in clones with the individual proteins (KASHDAN et al. 2001).

Synergistic effects of Cry-toxins were also observed when these toxins were expressed in genetically modified plants. For instance HERMAN et al. (2002b) described synergistic effects of the Cry34Ab1 and Cry35Ab1 proteins on susceptible insects. BOULTER et al. (1990) described synergistic effects of the pea lectin and the cowpea trypsin inhibitor towards *Heliothis virescens* when expressed in transgenic tobacco. Lowest insect survival was observed for both toxins expressed while the cowpea trypsin inhibitor alone was less toxic than the pea lectin when expressed individually in the transgenic plants (BOULTER et al. 1990).

GM plants expressing the Cry1Ac proteins and the cowpea trypsin inhibitor CpTI were reported to be less resistant towards four lepidopteran pests compared to the GM plant expressing the Cry1Ac toxin alone (SANTOS et al. 1997). The authors considered this result as a consequence of the Cry1Ac copy number although they did not exclude antagonistic interactions between the products of the two resistance genes.

13.2.5 Data on toxicity and specificity of bacterial proteins (Cry-proteins)

13.2.5.1 Presumed specificities of Cry-proteins

Several microbial pathogens are known to have a wide host range such as *Metarrhizium anisopliae* or *Beauveria bassiana* (more than 50 insect species in 7 insect orders). In contrast, the specificity of bacterial Cry-proteins of a certain class is considered rather narrow, being specific against a certain insect order. According to the classification system of HÖFTE & WHITELEY (1989) the distinction of the four major classes of Cry-toxins (CryI, CryII, Cry III and CryIV) was based on the gene structure of the resulting proteins and their insecticidal activity against Lepidoptera (CryI), Lepidoptera and Diptera (CryII), Coleoptera (CryIII) or Diptera (CryIV). However, as new genes with homology to known genes were shown to possess different insecticidal activity, the new nomenclature according to CRICKMORE et al. (1998) was introduced. The full list of toxins currently described and their classification can be obtained by CRICKMORE et al. (2007). As the current classification is sequence-based rather than function based it does not allow the prediction of the insecticidal activity of a certain new protein (CRICKMORE et al. 1998).

13.2.5.2 Indications of a wider specificity of Cry-proteins than previously assumed

As mentioned above the original classification of Cry-proteins was also based on their insecticidal specificity as reported by HÖFTE & WHITELEY (1989). However, the toxicity data for these Cry-proteins were based on the knowledge of their insecticidal effects to a small range of insects, most of which have or had, at least in some regions, agronomic relevance or even pest status. For example, the toxicity data provided for the activity of the Cry1-proteins by HÖFTE & WHITELEY (1989) comprised five lepidopteran species, all of agronomic relevance (*Pieris brassicae*, *Manduca sexta*, *Heliothis virescens*, *Mamestra brassicae* and *Spodoptera littoralis*). Similarly, specificity for the Cry2-proteins was based on tests with a few lepidopteran (*Heliothis virescens*, *Lymantria dispar*, *Manduca sexta*) and one dipteran larvae (*Aedes aegypti*; HÖFTE & WHITELEY 1989). Toxicity of the Cry-proteins of the third class (Cry3) and fourth class (Cry4) were based on insecticidal activity against the Colorado potato beetle (*Leptinotarsa decemlineata*) and certain mosquito species, respectively (HÖFTE & WHITELEY 1989). This focus on the use of a few, agronomically important “target” insect species for the determination of the “host range” and “relative efficacy” of individual **Bt** proteins is also reflected in more recent publications such as those of MACINTOSH et al. (1990) or SIMS (1995). For instance, SIMS (1995) used four “target” lepidopteran species and 10 non-lepidopteran species for acute toxicity tests. Only four out of the 14 species tested are beneficial species without any agronomic relevance as a pest species (*Apis mellifera* and *Nasonia vitripennis*; Hymenoptera; *Chrysopa carnea*, Neuroptera, *Hippodamia convergens*, Coleoptera).

Although specificity of a certain Cry-protein is considered to be largely restricted to a certain insect order, some Cry-proteins were found to be active against more than one insect order. For example, the Cry2A and Cry1Ab7 proteins are active against Lepidoptera and Diptera and the Cry1Ba protein is known to be active against Lepidoptera, Coleoptera and Diptera (see references in ZALUNIN et al. 2004). Also it is recognized that within a certain insect order, the sensitivity against a specific Cry-protein may differ considerably.

GM plants and unexpected effects on non-target organisms

Due to the assumption that Cry-proteins are specifically toxic to a certain insect order, effects of a particular Cry-protein on insects other than the presumed target organism were considered as “unexpected”. However, in the literature a range of indications can be found that Cry-proteins exert effects on other organisms than the target species.

HILBECK & SCHMIDT (2006) reviewed peer-reviewed studies on non-target invertebrates since 1995. Species evaluated were mostly herbivores (24 species from six insect orders) and 25 predator species from four insect orders as well as two herbivorous and three predatory mites and one spider species were tested. A few soil organisms and one aquatic species were examined in non-target studies (HILBECK & SCHMIDT 2006). They found that in half of the studies some kind of adverse effect on one or more of the parameters tested were reported.

The reports on adverse effects of the lepidopteran-specific **Cry1Ab or Cry1Ac toxins** on lepidopteran species other than the pest species targeted were surprising although earlier studies had already shown differences in susceptibility of various lepidopteran species (MACINTOSH et al. 1990, SIMS 1995). First evidence for effects on non-target butterflies came from the studies of LOSEY et al. (1999) reporting adverse effects of Bt176 corn pollen on the non-target monarch butterfly. Later such effects of Bt176 corn pollen on non-target butterfly larvae were confirmed by JESSE & OBRYCKI (2000), STANLEY-HORN et al. (2001), ZANGERL et al. (2001) and others (FELKE et al. 2002, FELKE & LANGENBRUCH 2003 and SHIRAI & TAKAHASHI 2005). Adverse effects from corn pollen of other events expressing the Cry1Ab toxin, especially after prolonged exposure were reported by DIVELY et al. (2004) and effects from corn anthers were reported by ANDERSON et al. (2004) as well as combined effects from corn pollen and anthers by ANDERSON et al. (2005). The Cry1Ac toxin expressed in **Bt** corn pollen was shown to be less toxic to non-target lepidopteran monarch larvae than the Cry1Ab toxin but sublethal effects were comparable (HELLMICH et al. 2001).

Also negative effects of the Cry1Ab or the Cry1Ac protein on other insect orders than Lepidoptera were reported. The early reports of adverse effects of the Cry1Ab toxin to green lacewing larvae, either in bi- or in tritrophic experiments (HILBECK 1998a, 1998b, 1999) were controversially discussed (DUTTON et al. 2002, ROMEIS et al. 2004, RODRIGO-SIMON et al. 2006). Feeding of **Bt**-fed lepidopteran larvae resulted in lower survival of carabids (MEISSLE et al. 2005). Adverse effects on coccinellids have been reported either when feeding directly on Cry1Ab toxin solutions or in tritrophic feeding trials (SCHMIDT et al. 2004). Several sublethal effects of **Bt** leaves when fed to detritivorous isopods have been observed (ESCHER et al. 2000, WANDERLER et al. 2002, PONT & NENTWIG 2005). Negative effects on parasitoids when developing in **Bt**-fed (Cry1Ab) host species were reported from a range of studies (e.g. PRÜTZ & DETTNER 2004, MEISSLE et al. 2003, VOITECH et al. 2005). Negative tritrophic effects of Cry1Ac expressing cotton were reported for some predatory bugs feeding on Lepidoptera (PONSARD et al. 2002) and for parasitoids (BAUR & BOETHEL 2003).

Adverse effects of the coleopteran specific **Cry3Bb1 toxin** on non-target organisms such as a coccinellid species were reported resulting in higher mortality of the coccinellid although this effect was not observed in a tritrophic feeding trial (SCHMIDT et al. 2004). Effects of coleopteran specific **Cry3A toxins** on other insects than Coleoptera were reported by DEML et al. (1999) who reported a distinct toxicity of the Cry3A protein on different lepidopteran larvae comparable with the Cry1Ac protein. The authors also reported only low toxicity of the Cry3A protein to the target coleopteran larvae **Leptinotarsa decemlineata** thus questioning the effectiveness of this toxin against coleopteran pests. Also tritrophic effects of this Cry-toxin on parasitoids were reported (ASHOURI et al. 2001). Reports on reduced growth and fertility of a lepidoteran species **Spodoptera littoralis** when fed Cry3A-expressing potato foliage or artificial diet were recently described by HUSSEIN et al. (2005).

Possible explanation of effects on non-target organisms

Two major explanations have been suggested for the observation of negative effects on insect species which are not considered to be the “target species”. Indirect effects either due to reduced nutritional quality of the prey species or differences in the plant

composition between the GMP and non-GMP. Direct toxic effects are rarely considered in order to explain adverse effects of specific Cry-toxins on non-target organisms.

Reduced nutritional prey quality is mostly used as an explanation for the observed adverse effects on non-target organisms. The negative effects on the green lacewing larvae observed in tritrophic experiments were attributed to lower nutritional quality of the intoxicated prey fed to the larvae and not to direct toxic effects of the **Bt** toxin by some authors (DUTTON et al. 2002, ROMEIS et al. 2004, RODRIGO-SIMON et al. 2006) although this cannot explain the direct toxic effects observed in some other studies. Other authors considered it unlikely to be the cause for the observed effects and suggested the possibility of another mode of action of **Bt** toxins in these organisms or complex interactions of the **Bt** toxin with, e.g. with plant secondary metabolites (HILBECK & SCHMIDT 2006).

Observed adverse effects on parasitoids were explained by host-mediated effects such as reduced survival or food consumption rather than a result of direct toxicity (PRÜTZ & DETTNER 2004, MEISSLE et al. 2003, VOITECH et al. 2005). Changes in host quality were considered the most likely explanation of adverse effects of **Bt** on parasitoids although direct effects of the toxin were also not excluded in some cases (ROMEIS et al. 2006b). Effects on a coleopteran predator were largely attributed to changes in tissue composition in the host although the authors cannot exclude direct toxic effects or a combination of both, a direct and an indirect effect (e.g. MEISSLE et al. 2005).

Differences in plant composition were suggested to be the likely cause for observed adverse effects on soil organisms (ESCHER et al. 2000, WANDELER et al. 2002, PONT & NENTWIG 2005).

Direct toxic effects of the Cry-toxin were in most cases excluded as an explanation for the effects observed although direct evidence is rarely given. In the case of the highly controversial effects on the green lacewing larvae a lack of binding of the Cry-toxin has been reported which is used to argue that the effects observed are due to indirect prey quality effects (ROMEIS et al. 2006b). Only in the case of affected lepidopteran species other than target Lepidoptera direct effects underlying the same mode of action as in target lepidopteran larvae have been suggested due to the close taxonomic relation between the target and the non-target species. However, the exact mechanism still seems to be unclear because the reported effects of the Cry- toxins on a specific non-target lepidopteran species such as *Spodoptera littoralis* ranged from lethal and sublethal effects to no or little effects (see discussion in HILBECK & SCHMIDT 2006). As an explanation for the observed differences in susceptibility between lepidopteran species also the “natural variation” has been mentioned. Later differences in the susceptibilities even between populations of a particular lepidopteran pest species were recognized which can vary up to 17.5 fold (e.g. KRANTHI et al. 2004).

Authors who did not report any adverse effects of the Cry3Bb1 toxin on coccinellid species suggested that the toxin is not activated, does not bind to the insect's gut or that the toxin may be altered in the gut (LUNDGREN & WIEDENMANN 2002).

13.2.6 Data on toxicity and specificity of plant proteins

Plant proteins expressed in GMPs such as the snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) are considered to have a broader specificity towards insects than Cry-proteins. As has been shown GNA-expressing potatoes exhibit negative effects on aphids, thrips and spider mites (ZEMKOVÁ ROVENSKÁ & ZEMEK 2006) but also on lepidopteran pests such as the tomato moth, *Lacanobia oleracea* (GATEHOUSE et al. 1997). Coleopteran species (*Adalia bipunctata*; Coccinellidae) were affected in tritrophic trials with GNA-expressing GM potatoes (BIRCH et al. 1999) although this has not been confirmed in another study (Down et al. 2003). Furthermore, parasitic wasps were also

negatively affected (ROMEIS et al. 2003a) and effects on soil communities (GRIFFITHS et al. 2000) and on planthoppers (POWELL et al. 1995) were reported.

Also proteinase inhibitors (PIs) are considered to have a wider specificity range than **Bt** toxins (O'CALLAGHAN et al. 2005). Specificity of PIs was attributed to the predominant proteases present in the insect's gut as they constitute the target enzymes of the PI. Consequently, serine PIs are considered effective against Lepidoptera whereas cysteine PIs are effective against some Coleoptera. Cysteine PIs were also shown to negatively affect aphids as cysteine-type proteases are the major endoproteolytic components in the aphid digestive tract (HAQ et al. 2004).

HILDER et al. (1987) were the first to show the effectiveness of a trypsin inhibitor in GM tobacco against a wide range of insect pests, including Lepidoptera, Coleoptera and Orthoptera. Following this study, several other PIs were used for the expression in GMPs also targeting nematodes or aphids (see overview in HAQ et al. 2004). In some cases PIs have also been shown to harm pollinators such as honey bees and bumble bees when ingested at high concentrations or natural enemies and parasitoids (see review in O'CALLAGHAN et al. 2005). Under field conditions a lower abundance of Collembola was recorded associated with GM tobacco leaves expressing cysteine PIs (DONEGAN et al. 1997).

13.3 Proteins are rapidly degraded in the soil environment

13.3.1 Hypothesis of the rapid degradation of Cry-proteins in the soil environment

The safety of plant-incorporated Cry-toxins to the environment is frequently argued with the limited persistence of these toxins in the soil environment and/or their ubiquity in the environment (see Section 12.2.4.3).

13.3.2 Evidence for the persistence of Cry-proteins in the soil environment

Cry-toxins can be released into the soil via degradation of plant material present in the field after harvest, via pollen dispersal or via root exudates during plant growth. It has been shown that the Cry-toxins can bind to clay minerals and humic acids or clay-humic acid complexes from soils thus reducing their availability to soil microbes (see review in STOTZKY 2004).

It has remained controversial how long these toxins can persist in the soil environment and remain insecticidal. Estimates on persistence and larvicidal activity of Cry-toxins in the soil vary although the majority of the studies conducted so far demonstrated persistence of insecticidal Cry-proteins over at least some time. The persistence of insecticidal parasporal crystals of **Bt** in soil has been estimated for up to 1-3 months (PRUETT et al. 1980, WEST 1984) or of sporular *Bacillus thuringiensis* formulations with more than 6 months (TAPP & STOTZKY 1998). PALM et al. (1994) were able to detect either purified Cry-toxins or derived from **Bt** cotton tissues in soil until the experimental end of 30 days. Later, the same authors report toxin detection up to 140 days (PALM et al. 1996) and others reported a similar period (120 days; SIMS & REAM 1997). Also industry-conducted studies showed that the toxin was detectable at the end of the experimental period (30 days, SIMS & HOLDEN 1996).

Prolonged persistence and insecticidal activity of Cry-toxins especially when bound on surface active soil particles thus being resistant to microbial degradation was confirmed

by several authors. Persistence has been reported for up to 180 days (SAXENA & STOTZKY 2002), more than 234 days (TAPP & STOTZKY 1998), up to 7-8 months (ZWAHLEN et al. 2003a), up to 200 days (ZWAHLEN et al. 2003b) or 7 months (BAUMGARTE & TEBBE 2005). In contrast, other authors report rapid degradation of the Cry-toxins and persistence for a few days only (HOPKINS & GREGORICH 2003, DUBELMANN 2005, HERMAN et al. 2001, 2002a, c).

Evidence for prolonged persistence of proteins comes also from other proteins than Cry-toxins such as proteinase inhibitors expressed in transgenic plants. In GM plant litter the proteinase inhibitors were still detectable after 57 days in soil (DONEGAN et al. 1997).

Several parameters have been shown to affect toxin binding and persistence of Cry toxins in the lab and in the field and are consequently influencing the experimental results in the above-mentioned studies. Such parameters are:

- The part of the plant examined, e.g. roots versus leaves (BAUMGARTE & TEBBE 2005).
- The extent of physical destruction of the plant material used in experiments (intact versus ground leaves).
- Soil composition, in particular clay concentration and type of clay minerals (montmorillonite, kaolinite; SAXENA & STOTZKY 2002).
- Physiochemical soil characteristics such as pH and moisture (TAPP et al. 1994, TAPP & STOTZKY 1998).
- The positioning of the plant material in the experimental setting (top of soil or incorporated into soil, ZWAHLEN et al. 2003a).
- Environmental conditions, in particular temperature (ZWAHLEN et al. 2003b).
- The initial concentration of the toxin at the beginning of the experiment (CLARK et al. 2005).
- The lignin content of the plant (SAXENA & STOTZKY 2001) and therefore the protein source used (purified versus plant-incorporated).

14 Critical appraisal of methods used in the ecotoxicity assessment of GM plants proteins

14.1 The risk assessment approach

In principle the tiered risk assessment approach has been recognised as an appropriate approach to assess risks of GMPs to non-target organisms (ANDOW & HILBECK 2004, ROMEIS et al. 2006a, b).

ANDOW & HILBECK (2004) criticise the use of the laboratory studies with isolated transgenic products as the sole basis for decision making in non-target risk assessment of GMPs. The authors argue that laboratory studies derived from ecotoxicity concepts used for chemicals have limited relevance with respect to the selection of species, the endpoints considered, and test methodology chosen. They emphasize the need for long-term complex field assessments in order to take complex interactions of the GMP and the environment into consideration (ANDOW & HILBECK 2004). Instead an ecological model for the risk assessment of GM crops which is based on ecological principles, considering the transgene, the organism and the relevant environment is proposed (ANDOW & HILBECK 2004).

DUTTON et al. (2003b) recommend using existing tiered testing approaches for plant protection products as specified in US and EU regulation but with some modifications. They consider the use of well-designed laboratory studies being able to elucidate or exclude negative effects on non-target organisms in the field and with no further testing requirements if first tier laboratory tests reveal no effects for a given species. If hazards are detected at lower tier tests, further lower tier laboratory tests may be used thus avoiding the costs and uncertainties of higher tier testing (ROMEIS et al. 2006a). DUTTON et al. (2003b) recommend a tiered testing system which starts with a first tier test establishing sensitivity of a given species using the insecticidal protein and the whole GMP, then proceeding to a second tier test to assess toxicity at exposure levels which are more representative of field conditions allowing movement of the selected species and using the whole plant. While endpoints of the first tier are LC_{50} , NOEC or NOEL the type of endpoint chosen in the second tier is left open and depends on the results of the first tier. Field tests are established in the third tier testing, if specific risks cannot be addressed in lower tier tests (DUTTON et al. 2003b).

14.2 Selection of species in ecotoxicological testing

Test organisms used in toxicological testing of GMPs in notifications for cultivation within the European Union are usually restricted to a few taxa. The set of species chosen is derived from testing requirements for plant protection products in the US. These species seem to be chosen for practical reasons as they are easily maintained under laboratory conditions but are not representative of the environment in which the GMP will be grown (see also O'CALLAGHAN et al. 2005).

The use of universal indicator species and safety factors in order to extrapolate from indicator species to all other species has been criticised as it does not correspond to the case-by-case approach required for a particular GMP and a particular environment (ANDOW & HILBECK 2004). Instead the authors proposed that species selection should follow several steps: establishment of functional groups according to their ecological role in the ecosystem, classifying the non-target organisms into these functional groups, species prioritization and selection of species of high priority.

Furthermore, HILBECK (2004) suggests applying five criteria in order to select species to be tested: co-occurrence in the target agro-ecosystem, abundance, association with the target crop, trophic linkage with the crop or their natural enemies, and significance to humans. The same range of criteria can be applied for soil organisms including abundance and functional significance (HILBECK 2004).

With respect to the selection of test species used in laboratory toxicity studies, DUTTON et al. (2003b) suggest the use of economically and ecologically relevant species important for natural pest regulation in the target crop and according to the likelihood of their exposure to the insecticidal protein but also taking a broad range of taxonomic arthropod groups into consideration. ROMEIS et al. (2006a) suggest the use of surrogate species, representing different ecological functions (herbivory, pollination, predation, parasitism, decomposition). Thus the data gained from lower tier tests can be transferred to a wide range of regions and both annual and perennial crops (ROMEIS et al. 2006a). Some doubts were raised about the inclusion of target (ROMEIS et al. 2006b) or non-target herbivores (DUTTON et al. 2003b) in test regimes as they are either the target of the plant protection measure (the GMP) and natural enemies will switch to other species if their prey is scarce (ROMEIS et al. 2006b) or due to the fact that they are also not included in risk assessments requirements for pesticide products (DUTTON et al. 2003b). The need to consider several major functional groups has been also emphasized by other authors, including non-target herbivores as they provide invaluable ecological services such as weed suppression, pollination and decomposition (LOSEY et al. 2004).

In recent years species of value for nature conservation or of aesthetic value have been the focus of risks of GM plant pollen to non-target lepidoptera. In the US the monarch butterfly was subject to several studies evaluating effects of GM maize pollen on this species and later evaluating exposure of monarch butterfly populations and finally estimating risk for the whole population. Also in Japan lepidopteran species that are endangered or vulnerable were evaluated for their possible exposure to **Bt** maize pollen (MATSUO et al. 2002 in SHIRAI & TAKAHASHI 2005). As a species selection criteria the significance to humans of a certain species has been suggested by HILBECK (2004).

14.3 Parameters assessed in ecotoxicological testing

The primary parameters assessed in ecotoxicity risk assessments are mainly acute response parameters after short-term exposure (see also Section 12.2.4.2). However, these parameters give little information on effects at a population, community or ecosystem level or for long-term effects (ANDOW & HILBECK 2004). Short-term experiments, such as 14 days for earthworms or 9-30 days for ladybird beetles have been criticised as they do not reflect the pattern and duration of exposure of these organisms under field conditions (MARVIER 2002). The life span of such test organisms ranges up to several months or even years therefore prolonged exposures should be requested (MARVIER 2002).

It has also been proposed to use toxicological endpoints related to fitness such as generational fitness (e.g. relative lifetime survival and reproduction). Survival should be evaluated at least through one generation including all the immature stages as well as adult life-stage parameters such as mortality and female fecundity. Test duration should be coupled with the exposure in the field (ANDOW & HILBECK 2004).

The difficulty of assigning a role for the fitness of the test species to a specific response variable is emphasized by LÖVEI & ARPAIA (2005). They consider physiological and behavioural parameters as more sensitive in showing impacts than mortality or population growth rate or refer to the evaluation of an “accumulated impact” over time such as progeny viability. Also the use of immature and therefore usually more sensitive stages which have narrower tolerance limits than adults has been proposed (LÖVEI & ARPAIA 2005).

Life table parameters such as survival or mortality, development and reproduction have been suggested as appropriate endpoints for risk assessment studies by some authors (DUTTON et al. 2003b, ROMEIS et al. 2006a) although multiple life stage testing was also recommended by ROMEIS et al. (2006a). Behavioural tests such as feeding preference tests have also been suggested as relevant as shown for the green lacewing larvae (DUTTON et al. 2003b).

14.4 Test methodologies applied and test substances used in ecotoxicological testing

The experimental design of laboratory ecotoxicological testing should reflect the conditions in the field as much as possible with respect to pattern, duration and extent (dosage) of exposure (MARVIER 2002).

Test substances usually used in toxicological tests are the microbially produced proteins incorporated into artificial diets. Functional differences between proteins expressed in microbes and in plants may be due to amino acid sequence differences or differences in the treatment of the protein.

Differences between the sequence of the Cry35Ab1 protein in the plant and the respective protein when expressed in a bacterial host were detected (GHAO et al. 2004). The expression of the protein in a microbial host followed by trypsinization can result in different protein fragments and influence protein function (see also 12.2.1).

Trypsinization of microbially produced proteins can result in significantly different mortalities in the target organism as shown in the case of Cry34Ab1/35Ab1 protein (GAO et al. 2004).

The artificial diet and the administered protein might not be ecologically relevant. The isolation of the product from the plant in which it is expressed and therefore excluding its role in several plant functions has been identified as the major drawback of the use of isolated gene products in toxicological tests (ANDOW & HILBECK 2004). According to DUTTON et al. (2003b) both, pure insectidal proteins incorporated into artificial diets and transgenic plant material, should be used. If appropriate artificial diets are not available, dose-response tests could be done by feeding prey species reared on artificial diet with the insecticidal protein at various concentrations (DUTTON et al. 2003).

It has been shown that the experimental design used in the toxicological tests provided by the notifiers is limited to detect significant effects (MARVIER 2002). According to the author's calculations, only one out of five notifier studies included a sufficient number of replicates to a 50 % reduction in survival of organisms exposed to the Cry-protein and none was able to detect a 20 % difference between the treatments. Although the effect size to be detected in these tests may be a policy decision, it is clear that the limited sample size and replication used in these tests as well as the lack of a power analysis does not allow a scientifically rigorous conclusion on the risk for a certain non-target organism.

Testing protocols used for ecotoxicological testing of GMPs are rarely standardized. Protocols for the assessment of effects of pesticides on non-target organisms have been considered as useful by some authors as they allow the transfer of test results among laboratories, countries and, if appropriate, across crops (ROMEIS et al. 2006a). A modified test methodology from the ecotoxicological model including long-term exposures of the transgene product as well as a whole-plant method allowing for the evaluation of several potential exposure pathways was proposed by ANDOW & HILBECK (2004). Additionally, key criteria for testing methodologies for initial testing approaches relating to the food offered, life stages tested, use of plants or part of plants, scientific

control and replication were suggested by these authors. Exposure of selected natural enemies in test systems should be represented in an ecologically relevant way, but at higher than typical concentrations (HILBECK 2004). This makes it necessary to characterise the material to which the organism is exposed. If necessary an assessment in bi- or tritrophic systems should be done (HILBECK 2004).

The problem of the lack of ecological realism in laboratory tests with non-target organisms has been emphasized by LÖVEI & ARPAIA (2005) as the laboratory conditions are unable to reflect the additional stresses for a non-target species encountered in the field. The authors recommend the inclusion of some of the important stressors of field conditions such as prey shortage, variable temperatures or mixed feeding.

14.5 Methods used for assessing persistence of Cry-toxins

In many cases experimental methods applied in GMP notifications for the evaluation of the persistence of Cry-proteins may not be suitable with respect to the effectiveness of toxin recovery, the underlying model of the decay and the insects and methods used in the bioassay when determining the larvicidal activity of Cry-toxins in soil (see also Section 12.2.4.3).

It has been shown that toxin recovery from soil varies with the method and the soil type used in experiments. Toxin recovery is a function of the initial toxin concentration in the soil and especially at low toxin concentrations poor recovery rates were reported. For instance PALM et al. (1994) reported recovery of purified toxin from three different soils ranging from 27 to 60.2 % and for GMP toxin from 17–70 %. They concluded that several extractions were necessary in order to recover at least 90 % of the toxin. Also BAUMGARTE & TEBBE (2005) reported less than 40 % toxin recovery for two types of soil and found that one specific soil type yielded significantly higher amounts of the toxin.

Data generated from toxin estimations for the evaluation of soil persistence do not fit in all cases to any exponential decay curve in order to estimate the half-lives of the toxin (DT_{50}). PALM et al. (1996) found that only in four of eleven treatments the data fitted an exponential decay curve. They suggest a two step rate of decay, beginning with a rapid decline of the toxin concentration and then a more gradual rate of decline is observable. Consequently, a rather non-exponential function has been proposed in order to describe Cry-toxin decay rates (PALM et al. 1996). This was also confirmed by DUBELMANN et al. (2005) who used only the first four time points of the growth inhibition data to calculate a half life of the Cry-toxins because the whole data did not fit an exponential curve. It has been doubted whether the frequently used half-life concept (DT_{50}) developed for the decay of radionuclides is applicable to protein degradation which do not decay at a constant rate but differ between free and bound proteins (STOTZKY 2004). Suggestion were made for a bi-exponential equation or bi-phasic model to describe decay rates of Cry-toxins in a more realistic way (WOLT et al. 2000 cited in DUBELMANN et al. 2005). This model assumes that the decaying material partitions into two compartments within the soil – the sorbed and the one in solution – and that the decay of each component differs. DUBELMANN et al. (2005) used a linear explanatory model (shift-log model) which does not assume any specific mechanism underlying the decay.

It has been observed that decay rates vary considerably between experiments. For instance, even under the same laboratory conditions it was observed that less than 0.1 % up to 85 % of the initial toxin concentration remained after 28 days (PALM et al. 1996). The authors explain these differences with differences in soil type, plant material used and temporal and seasonal variations in soil qualities (PALM et al. 1996).

Furthermore, decay rates of purified toxin and GM plants have been shown to differ considerably. PALM et al. (1996) report faster degradation of the **Btk** toxin in plant tissues than the purified toxin which they explain by the presence of degradative enzymes and compounds released by the plants or by plant-associated micro-organisms.

Insect bioassays are frequently used in degradation studies in order to determine the larvicidal activity of the toxins remaining in soil. However, these bioassays are not standardized and the literature provides a range of studies, using different insect species even for the same Cry-toxins (*H. virescens*, *O. nubilalis* etc.), different larval stages, different periods of the bioassay (3 to 7 days) and different endpoints (mortality, weight inhibition) as an index of bioactivity.

In field experiments methods are even less consistent. Collection of soil material from fields where **Bt** maize was grown is usually done in a non-standardized way, often it is not indicated where the soil was collected. In one study, the authors refer to “**near individual corn plants but at a distance sufficient to minimize inadvertent collection of root tissue with the soil sample**” (DUBELMANN et al. 2005). The exact position of the soil sample taken may be important as bulk and rhizosphere soil may differ in their toxin content (see e.g. BAUMGARTE & TEBBE 2005). In several instances indication of the soil depth is lacking at which soil samples were taken. In other cases samples were taken at 15 cm (e. g. DUBELMANN et al. 2005) or at different depths (0–15, 15–30 and 30–60 cm, HOPKINS & GREGORICH 2003).

The above mentioned limitations show that the currently applied protein degradation studies in soil give only limited information on the fate and behaviour of Cry-proteins in soils. The use of whole GMPs instead of the purified protein as well as the inclusion of more than one soil type reflecting different soil environments with different clay concentrations as well as the use of biochemical parameters (soil bacterial profiles, enzyme assays and metabolic activities) could give a better insight into the fate of these proteins under field conditions.

15 Conclusions and Recommendations

The general **risk assessment approach** for proteins expressed in GMPs is inherently different from other regulatory contexts in the European Union such as microbial pesticides as it lacks the systematic risk assessment approach applied in other regulatory fields. As the European legislation for GMOs (Directive 2001/18/EC or Regulation (EC) 1829/2003) does not specify the risk assessment approach in such detail as required for other regulated products, a range of different approaches with respect to test substances, test species used and experimental setups is chosen by the notifying companies of GMPs. In contrast to GMOs the regulatory requirements for microbial plant protection products foresee guidelines with respect to test organisms, recommending testing guidelines and considering the fate, behaviour and persistence of these products in different environmental compartments including ground and surface waters. This procedure includes the commonly used approach of exposure and effects assessment for selected test organisms as well as the risk characterisation by determination of a toxicity-exposure ratio which is lacking in the risk assessment approach of proteins expressed in GMPs. Also trigger values for authorisation are specified for the registration of microbial pesticides but lacking in GMO legislative requirements. Although the risk assessment approach according to Directive 2001/18/EC is intended to be much broader than the US approach, notifiers for GMPs in the EU generally adhere to US testing requirements by submitting the studies required for deregulation of plant-incorporated protectants by the US Environmental Protection Agency.

Ecotoxicity assessment of Cry-proteins expressed in GMPs is based on a range of underlying **assumptions** with respect to the mode of action, the specificity and effects of Cry-proteins. It is presumed that Cry-proteins only act as acute toxins against the designated target insect pest species via a well-known mechanism. Non-target organisms are considered not to be exposed to the toxin or not to be susceptible to the toxin. The validity of some of these basic assumptions in the ecotoxicity assessment of GMPs as stated by the notifiers has to be severely questioned. There is evidence that the toxicity of a Cry-protein is dependent on its structure and the conditions of the ingesting organism as well as its digestive system. Several toxin-related, organism-related and environmental factors can modulate the toxicity and consequently the specificity of a Cry-protein. Consequently, there is a need to thoroughly review these underlying assumptions and more research is needed to clarify some of the fundamental questions of Cry-protein toxicity. Such research should not only focus on the presence of Cry-proteins in agriculturally relevant non-target organisms and habitats but also on the fate of these toxins in living organisms including degradation patterns and potential changes in specificity when passing through the food web.

In most cases of GMP notifications for cultivation in the EU a thorough **exposure assessment** is lacking. In the few cases where exposure analysis was done, its relevance is limited as it considers only direct exposure of non-target organisms via the GMP itself and is based on expression values of the introduced protein derived from oversea field trials not representative for European conditions. There is evidence that Cry-proteins are present in different environmental compartments such as agricultural soils and headwater streams. Cry-proteins are available to non-target organisms via several exposure pathways, either directly or indirectly after passage of lower trophic levels. However, the exposure of non-target organisms to GMP expressed Cry-proteins via several pathways generally remains unconsidered in GMP notifications. Therefore a thorough exposure analysis considering all relevant exposure pathways of the protein expressed in GMPs to non-target organisms, as required in other regulatory fields, would constitute a considerable improvement in the risk assessment approach of GMP expressed proteins.

The **assessment of ecotoxicological effects** of Cry-toxins to non-target organisms is derived from the regulatory requirements for ecotoxicological testing of microbial pesticides in the US with respect to the selection of test species and methodologies applied. The toxicological studies submitted by notifiers usually comprise a similar set of test species, exposed to a test substance expressed in a micro-organism and administered at a high dose and for a short period of time. This approach is derived from chemical testing and has limited relevance for the assessment of GMP expressed proteins. The use of a limited range of test species for ecotoxicological testing ignores the role of the specific environment in which a GMP is introduced and the protein-plant-environment interaction which is required by the case-by-case approach as outlined in Directive 2001/18/EC. Although the selection of relevant taxa for ecotoxicological testing and the criteria applied for selection are still controversially discussed it is clear that the current approach is severely limited.

Furthermore, this exclusively acute toxicity testing approach has limited relevance for species for which a life-long or even a multi-generational exposure to the protein expressed in the GMP can be envisaged. Ecotoxicity testing should comprise longer term exposure and should possibly be related to the actual exposure in the field. Also the use of repeated dose toxicity tests would give a better estimation for possible adverse effects of the protein in the field. The use of more sensitive life-stages of non-target organisms such as juvenile, diseased or parasitized test organisms would give an indication if the Cry-protein acts as an additional stressor in young or weakened organisms.

Test substances used in ecotoxicological tests are generally not representative for the exposure of non-target organisms in the field. There is evidence that in many cases Cry-proteins as expressed in plants are not identical to proteins expressed in bacteria with respect to their sequence, size and function. There is also evidence that small differences in amino acid sequence between proteins in general and specifically Cry-proteins can lead to differences in toxicity to certain organisms. Also the differential processing of proteins expressed in plants and in bacteria, either due to plant-derived enzymes or to treatment with enzymes before administration to the test diet yields different protein fragments that can result in differential toxicities. Finally, as the toxins constitute an additional stressor for the organism, secondary effects due to plant composition are not considered in studies with isolated proteins. Consequently, there is an urgent need to complement the currently used microbially expressed test substances by the relevant GM plant.

The validity of the ecotoxicological studies with non-target organisms and GMP proteins is largely not secured, as most studies are not carried out according to standardized **testing protocols**. Testing periods which are limited by the mortality of the control group cannot be considered as state of the art. Consequently, the use of relevant testing protocols is of high priority to ensure valid testing results. Standardized protocols for testing of microbial pesticides are currently available for a range of test species but would need to undergo a review process to ensure their suitability for the specific conditions of testing proteins expressed in GMPs.

The explanatory power of these tests is further reduced due to the fact that sample sizes are generally too low to detect other than very large differences in the parameters measured between test and control groups. Current risk estimations are based on too few replicates which make the detection of effects unlikely even if they would exist. Therefore there is an urgent need for sufficiently large sample sizes and power analyses to be included in the statistical evaluation of ecotoxicological testing to guarantee that certain differences are actually detectable with the methodology chosen.

Currently, a specific assessment of GMPs expressing more than one Cry-protein is not considered to be necessary by the notifying companies as potential **interactions** between the relevant proteins are considered to be unlikely. However, as the published

literature shows, there is evidence that proteins can interact if expressed in the same plant. Interactions can be synergistic or antagonistic and merit specific investigation depending on the combination in the GMP and the proteins introduced.

The submitted **degradation studies** of Cry-proteins in soil have considerable shortcomings with respect to methodology used, the underlying models and the conditions applied in these tests. Cry-proteins have the potential for longer term persistence and bioactivity in soil and probably in other environmental compartments such as surface waters. The assessment of low-level, long-term persistence in the field can definitely not be met by the currently used short term laboratory studies. The inclusion of several soil types, whole GMPs instead of isolated proteins, the application of biochemical parameters and the standardization of methodology for laboratory studies supplemented by field studies would be crucial to give a clearer picture of the actual persistence of Cry-proteins in different environmental compartments.

Research needs have to be emphasized for several areas. As our current understanding of the mode of action of Cry-toxins in different organisms and taxa is still limited, further research is needed to determine the fate and behaviour of these proteins in distinct non-target organisms and their effect on the food chain. This is also valid for the presence and persistence of Cry-proteins in several environmental compartments. Currently only few published data are available indicating the presence of Cry-toxins in soil, water, sediments or organisms related to agricultural systems. Finally, the development of new test protocols will be crucial, specifically for testing relevant non-target organisms for which no established protocols can be used or adapted and for the determination of the fate of proteins derived from GM plants in relevant environmental compartments.

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The overall goal of this report is to review the currently applied approaches in toxicity and ecotoxicity assessment of novel proteins in the context of genetically modified plants and to identify and discuss possible weaknesses and limitations. This was done by a review of the toxicity and ecotoxicity assessment practice by notifiers, relevant guidance documents and the published literature.



Recommendations for possible improvements of toxicity and ecotoxicity assessment in the context of GM crops are discussed.



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