Eur. J. Biochem. **267**, 2046–2053 (2000) © FEBS 2000

Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth *Galleria mellonella*

Andreas C. Fröbius[1], Michael R. Kanost[2], Peter Götz[1] and Andreas Vilcinskas[3]

[1]*Institute of Zoology, Free University of Berlin, Germany;* [2]*Department of Biochemistry, Kansas State University, Manhattan Kansas, USA;* [3]*Systematic Zoology, University of Potsdam, Germany*

Three inducible serine protease inhibitors (ISPI-1, 2, 3) have been purified from larval hemolymph of greater wax moth larvae, *Galleria mellonella*, and characterized at a molecular level. These inhibitors were synthesized after larvae were injected with a yeast polysaccharide, zymosan preparation. ISPI-1,2,3 were active against various serine proteases including trypsin and toxic proteases released by the entomopathogenic fungus *Metarhizium anisopliae*. Precipitation by trichloroacetic acid and heat, followed by FPLC and HPLC separation steps were used for purification of the protease inhibitors from cell-free hemolymph samples. The molecular masses of purified proteins were determined by MS to be 9.2 kDa (ISPI-1), 6.3 kDa (ISPI-2) and 8.2 kDa (ISPI-3) with isoelectric points ranging between 7.2 and 8.3. The N-terminal amino-acid sequences of ISPI-1 and ISPI-3 are not similar to other known proteins, whereas that of ISPI-2 exhibits extensive similarity to known Kunitz-type protease inhibitors.

Keywords: anti-fungal; serine protease inhibitor; insect immunity; *Galleria mellonella*; *Metarhizium anisopliae*.

The insect humoral immune response is characterized by a rapid and transient synthesis of proteins with potent antibacterial and/or anti-fungal activity [1]. The humoral immune response of larvae of greater wax moth *Galleria mellonella,* comprises a remarkable increase of lysozyme and cecropin-like molecules within the hemolymph which also exhibit anti-fungal activity *in vitro* [1]. Entomopathogenic organisms have evolved mechanisms for suppression or avoidance of immune responses within the infected insect host and produce enzymes which help to extract nutrients from its body. Entomopathogenic fungi utilize proteolytic enzymes which enable them to infect susceptible hosts directly via the exoskeleton as the proteases digest cuticle proteins [2]. Extracellular proteases produced by *Beauveria bassiana* or *Metarhizium anisopliae*, entomopathogenic fungal species used worldwide to control insect pests, seem to participate in suppression of cellular immune responses within the hemolymph of infected *G. mellonella* larvae. Isolated plasmatocytes, immune competent hemocytes of this insect, exhibited *in vitro* reduced phagocytic activity, attachment and spreading when incubated with fungal proteases [3]. A number of proteases produced by *M. anisopliae* have been purified and characterized [4–6]. The capacity to produce suitable proteases in sufficient quantities probably determines the virulence of particular fungal strains [7].

Insensitivity of virulent strains of entomopathogenic fungi to lysozyme in hemolymph and tissues of the infected host is probably mediated by released proteases [8]. *M. anisopliae* proteases are toxic when injected into *G. mellonella* larvae [9]. However, inhibitors of toxic *M. anisopliae* proteases have been detected within the hemolymph of this insect and have been partially purified [10]. Inhibitory activity against fungal proteases increased within the hemolymph of *G. mellonella* in response to injected fungal cells and preinjected larvae exhibited prolonged survival after subsequent infection with *B. bassiana* [11]. This observation stimulated us to identify the protease inhibitors which are induced during humoral immune response in *G. mellonella*. We used the soluble contents of a zymosan (freeze-dried yeast cells) preparation which consists predominantly of fungal cell wall derived β-1.3 glucans, to elicit humoral immune responses in this insect which are equivalent to those observed after injection of other microbial agents [8].

In this paper we present a protocol for the isolation of three inducible, heat-stable, low molecular mass protease inhibitors from cell-free hemolymph of *G. mellonella* larvae [inducible serine protease inhibitor (ISPI)-1–3]. These inhibitors are active against proteases Pr1 and Pr2 produced by *M. anisopliae*. Two of the discovered molecules exhibit no similarity to known proteins (ISPI-1, ISPI-3) whereas the amino-acid sequence of ISPI-2 is similar to Kunitz-type protease inhibitors.

Correspondence to A. Vilcinskas, Spezielle Zoologie und Evolutionsbiologie, Universität Potsdam, Villa Liegnitz, Lennèstrasse 7a, 14471 Potsdam, Germany. Fax: + 49 331977 4861, Tel.: + 49 331977 4884, E-mail: andreasvi@t-online.de

Abbreviation: ISPI, inducible serine protease inhibitor.

Enzymes: bovine pancreatic trypsin (EC 3.4.21.4); bovine pancreatic chymotrypsin (EC 3.4.21.1); subtilisin Carlsberg (EC 3.4.21.62); proteinase K (EC 3.4.21.64); porcine pancreatic elastase (EC 3.4.21.36).

(Received 9 November 1999, accepted 7 February 2000)

EXPERIMENTAL PROCEDURES

G. mellonella larvae rearing, immunization and hemolymph collection

An inbred strain of the greater wax moth, *G. mellonella* was reared on an artificial diet (22% corn meal, 22% wheat germ, 11% dried milk, 5.5% dried yeast, 17.5% beeswax, 11% honey and 11% glycerol) at 31 °C in the dark. Last instar larvae (300–400 mg) were used as a source of hemolymph. Humoral immune responses were elicited by intrahemocoelic injection of the soluble components of a zymosan preparation. Twenty

milligrams zymosan A (Sigma) was suspended in 1 mL sterile physiological saline (172 mM KCl, 68 mM NaCl, 5 mM $NaHCO_3$, pH 6.1 adjusted with HCl), sonicated for 15 min and nonsoluble compounds were removed by centrifugation at 10 000 ***g*** for 5 min. Ten microliters supernatant per larva were injected using disposable 1-mL syringes and 0.4 × 20 needles (Luer) mounted on a microapplicator. To inhibit transcription or translation of genes encoding inducible proteins, either actinomycin D or cycloheximide (Sigma) was dissolved in the supernatant (100 $\mu g \cdot mL^{-1}$) prior to injection. Hemolymph samples were collected by puncturing the larvae ventrally with a sterile needle. Out-flowing hemolymph was transferred to chilled tubes (containing few crystals of phenylthiourea to prevent melanization) and centrifuged twice at 100 ***g*** for 10 min to remove hemocytes. Pooled supernatants were stored at −20 °C until needed.

Purification of *Galleria* serine protease inhibitors from immune hemolymph

Each purification started with 10 mL of immune hemolymph supernatant. Frozen aliquots of cell-free immune hemolymph were thawed, pooled to obtain 10 mL and diluted by adding 80 mL ice-cold Tris/HCl buffer (50 mM, pH 7.5), referred to as buffer A. The diluted hemolymph was subjected to trichloroacetic acid and heat precipitation as previously described [12]. The dialyzed and freeze-dried supernatant was dissolved in 6 mL buffer A and applied to a Resource-Q column (1 mL, Pharmacia) equilibrated with the same buffer. The flow-through fractions containing the inhibitory activity were collected, and the column was washed with 1 M NaCl. Collected flow-through fractions were concentrated using Centriprep-3-devices (Amicon) and speed-vac and applied to a Superose-12 gel filtration column, equilibrated and run at 0.3 $mL \cdot min^{-1}$ with 0.15 M NH_4HCO_3 using an FPLC system (Pharmacia). Fractions containing inhibitory activity were pooled, desalted and concentrated by Centricon-3 devices and freeze-drying. A Hi-Pore reversed-phase column RP-318 (4.6 × 250 mm, Bio-Rad) was used for final separation of the low molecular mass serine protease inhibitors. The column was eluted with a 60-min linear gradient from 18% to 38% acetonitrile in 0.1% trifluoroacetic acid (Pierce) at a flow rate of 1 $mL \cdot min^{-1}$. Eluting proteins were monitored at 215 nm. Fractions were frozen at −70 °C, freeze-dried to remove acetonitrile, and dissolved in 100 μL water.

Protease inhibitor assay

To measure inhibitory activity against trypsin, an azocoll assay adapted to microtiter plates was used as described previously [12]. In this assay, azo-dye bound to collagen as a nonsoluble substrate is liberated by proteolytic activity and measured with a microtiter plate reader at 492 nm. Bovine pancreatic trypsin (Sigma) was used to determine inhibitory activity against serine proteases in fractions obtained during purification. For each sample assayed (mean value of at least four readings) a plot of absorbance vs. dilution factor was constructed to calculate the dilution factor corresponding to 50% inhibition of trypsin activity (IC_{50}).

The inhibitory activity of isolated proteins against several serine proteases was screened as described recently [13] with minor modifications. Ten microliters inhibitor solution (0.1 $\mu g \cdot \mu L^{-1}$) was incubated with 10 μL each of protease solution in 0.1 M Tris/HCl-buffer, pH 7.5, 0.1 M NaCl, 1 mM $CaCl_2$ for 10 min at room temperature. Then 0.7 mL of an appropriate chromogenic substrate solution (50 μM in the same buffer) was added, and the residual proteolytic activity was monitored by measuring the change in A_{405} with time. The enzymes and their substrates used in the tests were: N-Tosyl-L-phenylalanine chloromethyl-treated bovine pancreatic trypsin (5 $ng \cdot \mu L^{-1}$) (Sigma) and *N*-benzoyl-Val-Gly-Arg-*p*-nitroanilide (Sigma); bovine pancreatic chymotrypsin (0.1 $\mu g \cdot \mu L^{-1}$) (Sigma) and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide

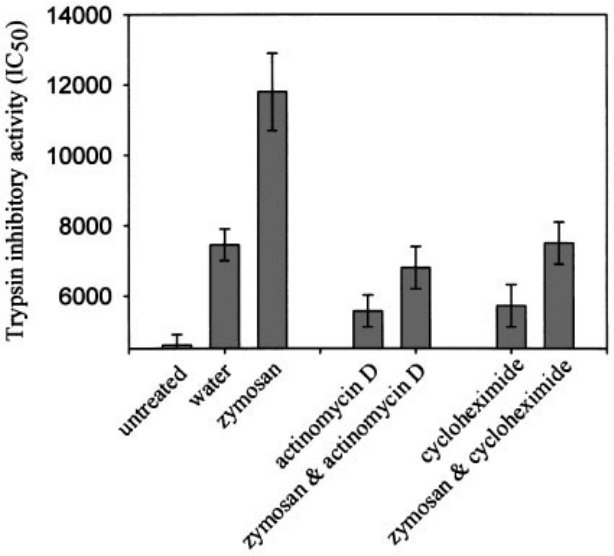

Fig. 1. Inhibition of inducible trypsin inhibitory activity in hemolymph of *G. mellonella* larvae by coinjection of a zymosan-preparation with specific inhibitors of protein synthesis. *G. mellonella* larvae were injected with zymosan with or without actinomycin D or cycloheximide and bled 12 h later. Untreated larvae or larvae injected with distilled water alone served as controls. Trypsin inhibitory activity was measured by the azocoll assay to calculate the dilution giving the IC_{50}. Data are given as mean ± SD for at least four measurements ($n \geq 4$).

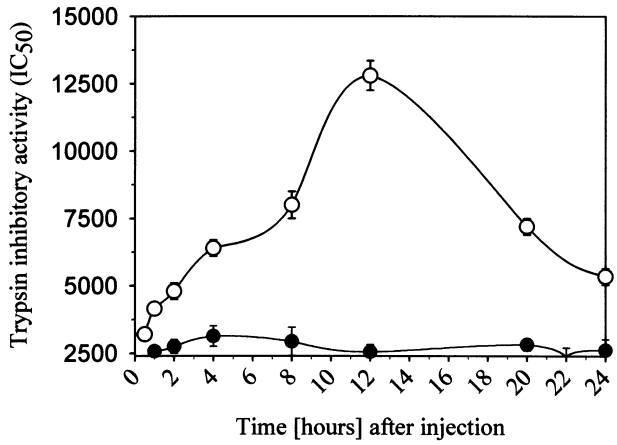

Fig. 2. Time-dependent increase of serine protease inhibitory activity in the cell-free hemolymph of last instar *G. mellonella* larvae in response to injected zymosan A preparation. *G. mellonella* last instar larvae were injected with the soluble components of a zymosan A preparation (20 $mg \cdot mL^{-1}$, 10 μL) and each group bled at different time intervals post injection. Trypsin inhibitory activity was measured with the azocoll assay to calculate the dilution giving the IC_{50} (○). Untreated larvae bled at the same time intervals served as controls (●). Data are given as mean ± SD for at least three measurements ($n \geq 3$).

(Sigma); subtilisin *Carlsberg* from *Bacillus licheniformis* (0.1 μg·μL^{-1}) (Sigma) and *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Sigma); proteinase K from *Tritirachium album* (0.1 μg·μL^{-1}) (Sigma) and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma); porcine pancreatic elastase (0.1 μg·μL^{-1}) (Sigma) and *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (Sigma; partially purified chymoelastase PR1 from entomopathogenic fungus *M. anisopliae* isolate ME1 (< 0.1 μg·μL^{-1}, 360 U·μL^{-1}) and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma); the trypsin-like proteinase PR2 from *M. anisopliae* ME1 (< 0.1 μg·μL^{-1}, 150 U·μL^{-1}) and *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Sigma). The proteases from *M. anisopliae* were gifts from J. P. Gillespie, Department of Biochemistry, University of Bristol, UK.

Protein determination

Protein concentrations in hemolymph samples and fractions obtained during purification were determined using the dye-binding-assay of Bradford [14]. BSA was used as the standard.

Determination of the number of cysteine residues

Ten microliters of a freeze-dried fraction obtained after reversed-phase HPLC was lyophilized and dissolved in 10 μL reaction buffer (250 mM Tris, 1 mM EDTA, 6 M guanidine-HCl, pH 8.5). After addition of 2.5 μL 2-mercaptoethanol (10%, v/v), the tube was flushed with nitrogen and the mixture incubated for 2 h at room temperature. Two microliters fresh

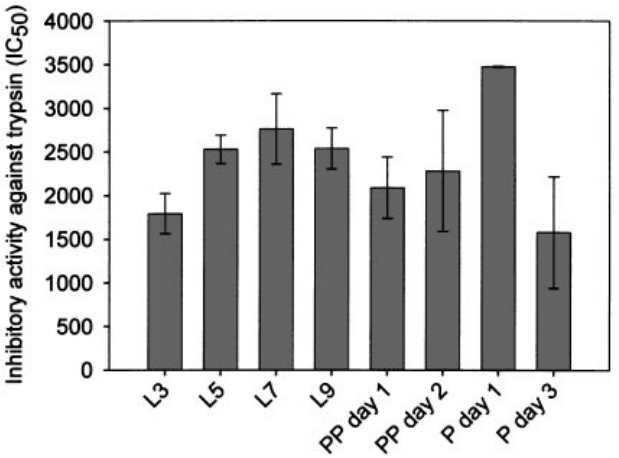

Fig. 3. Developmental changes of trypsin inhibitory activity in hemolymph of *G. mellonella*. Hemolymph samples were obtained from different larval stages (L3 to L9), prepupae (PP) and pupae (P) 1 or 3 d after pupation. Trypsin inhibitory activity was measured with the azocoll assay, to calculate the dilution factor providing 50% inhibition (IC_{50}). Data are given as mean ± SD for at least three measurements ($n \geq 3$). Trypsin inhibitory activity fluctuated in examined developmental stages, a significant increase of antitrypsin activity within the cell-free hemolymph was detectable in pupae 1 d after pupation.

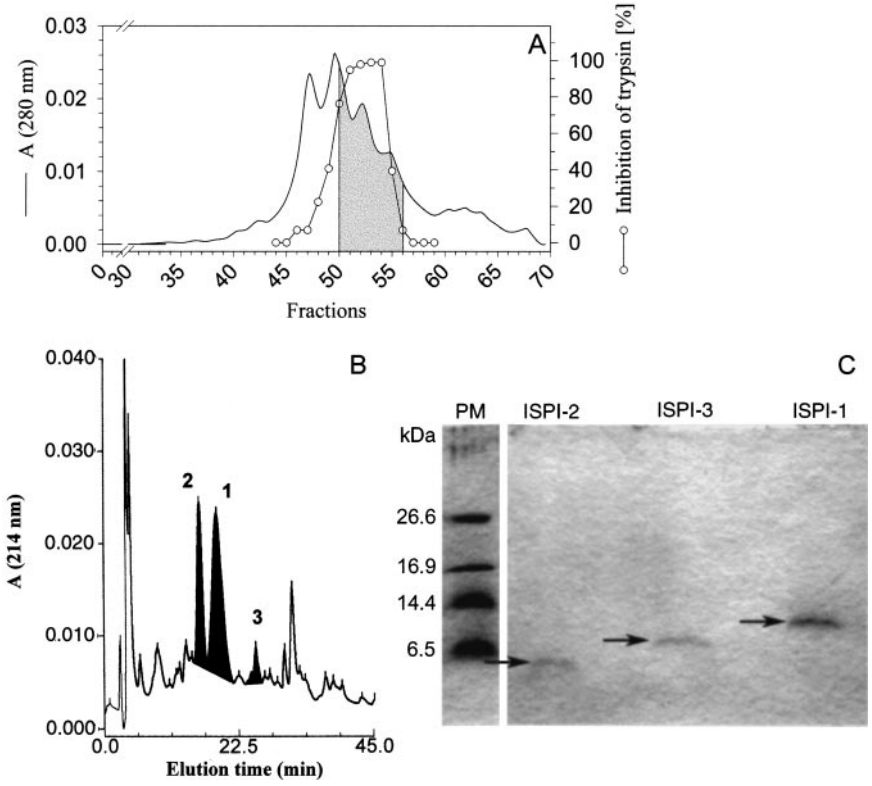

Fig. 4. FPLC separation of ISPIs from cell-free immune hemolymph of *G. mellonella* (A), reversed-phase HPLC separation of ISPIs (B) and fractions containing ISPI activity were subjected to electrophoretic analysis (C). Separation of enriched ISPIs achieved after trichloroacetic acid and heat precipitation (100-fold enrichment) of cell-free hemolymph by anion-exchange chromatography on a Resource Q column. Steady-state elution at pH 7.5 was performed: Trypsin inhibitory activity did not bind to the column. Concentrated Resource Q effluent (780-fold enrichment) was separated by gel filtration on Superose 12. The pooled and concentrated fractions 51–56 (shaded), containing proteins of ≤ 25 kDa, were used for further purification on HPLC. Absorbance during these steps was monitored at 280 nm. (B) The pooled and concentrated fractions 51–56 from the gel filtration step (2700-fold enriched compared to hemolymph) were applied to a Hi-Pore RP-318 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. Elution was performed using a linear gradient from 18 to 38% acetonitrile in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1 mL·min^{-1}. Absorbance was monitored at 214 nm. The shadowed peaks contained the ISPI activity. 1,2 and 3 correspond to the inhibitors ISPI-1, 2 and 3. (C) SDS/PAGE and Coomassie-blue staining documented the separation of three bands with molecular masses which correspond to the data obtained by MS. PM, peptide marker.

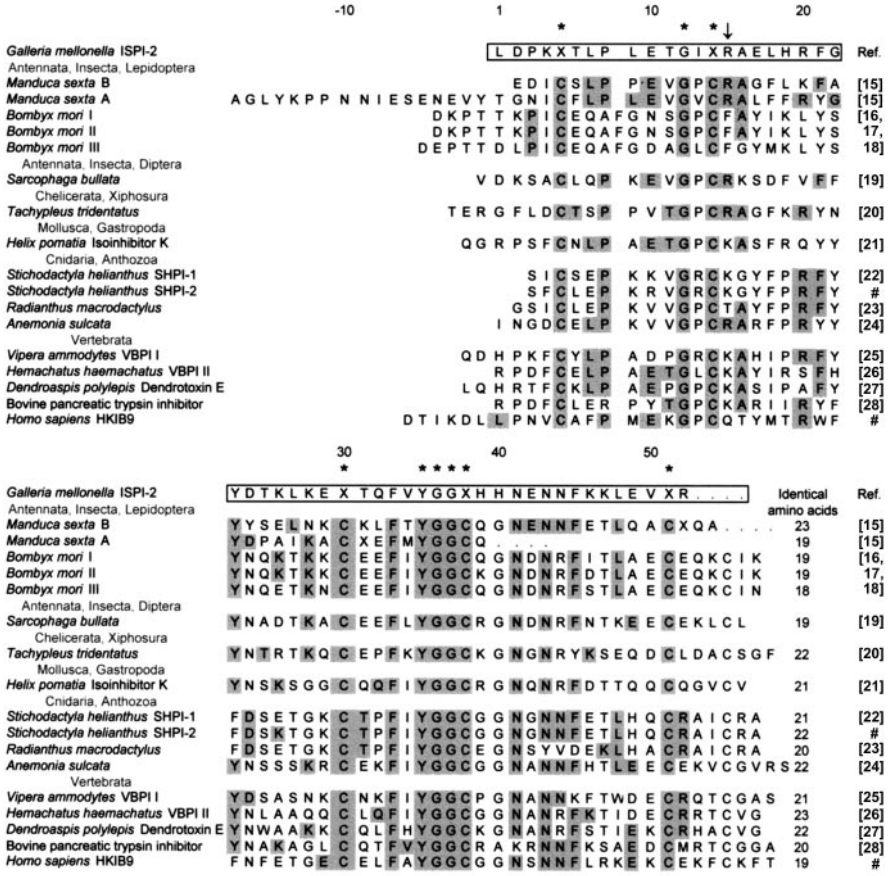

Fig. 5. Alignment of the amino-acid sequences of invertebrate and vertebrate bovine pancreatic trypsin inhibitor-Kunitz-type inhibitors with the sequence of the inducible serine protease inhibitor 2 (ISPI-2). The sequences of ISPI-2 and the inhibitors of *Manduca sexta* are incomplete. The mass of ISPI-2 (6337 Da) suggests that an additional four residues at the C terminus were not determined. Residues that gave no signal (marked X) are likely to be Cys based on their positions in the alignment. Residues conserved in all sequences are marked by an asterisk, the putative P1-residue is marked by a downward pointing arrow and amino acids that are identical to those of ISPI-2 are shaded. Sequences with '#' as references were taken from SWISS-Prot-database-entries lacking references.

4-vinylpyridine (Sigma) was then added, and the sample was incubated for another 2 h at room temperature in the dark. For quenching, 2.5 μL 2-mercaptoethanol were added. This sample was immediately desalted by reversed-phase HPLC and freeze-dried before submission to MS.

Enzymatic deglycosylation procedure

Approximately 1 μg freeze dried inhibitor was dissolved in 30 μL 20 mM sodium phosphate buffer, pH 7.2. One unit of N-glycosidase F and 0.5 mU of N-glycosidase A (Boehringer Mannheim) were added according to the manufacturer's protocol, and the mixture was incubated at 37 °C for 24 h. Enzymes and salts were removed by reversed-phase HPLC, and the processed inhibitor was submitted to MS.

MS and amino-acid sequencing

For determination of the masses of the purified inhibitors and mass shifts after 4-vinylpyridinylation and enzymatic deglycosylation, a Finnigan Lasermat 2000 (MALDI-TOF-MS) with α-cyano-4-hydroxy-cinnamic acid (Aldrich) as matrix was used. Automated amino-acid sequencing was performed by Edman degradation using a PerkinElmer Applied Biosystems Model 473A protein sequencer.

Table 1. Purification scheme for the low molecular mass ISPIs from the immune cell-free hemolymph of *G. mellonella*. The titer of inhibitory activity was determined as the dilution of the sample which provided the IC_{50} of trypsin (1.7 μg) in the azocoll microtitre plate assay. ND, Not determined. Because the amounts of the pure separated inhibitors in the HPLC eluate were too small to be determined by the Bradford protein assay and using the azocoll assay was not possible at that time, the exact results of this purification step were not calculated.

Fraction	Volume (mL)	Total protein (mg)	Inhibitory activity (titer)	Total inhibitory activity (titer·mL^{-1})	Specific activity (titer·mg^{-1})	Yield (%)	Purification (x-fold)
Cell-free immune hemolymph	10	800	3044	30 440	38	100.0	–
Trichloroacetic acid supernatant	85	7.8	107	9071	1163	29.8	30
Heat supernatant	85	5.9	103	8840	1498	29.0	39.4
After dialysis	90	3.5	97	8706	2487	28.6	65.5
Concentrated supernatant	6	2.1	1341	8046	3831	26.4	100.6
Resource Q column effluent	15	0.2	596	5960	29 800	19.6	782.2
Superose 12 column eluate	3.8	< 0.05	1382	5112	102 240	16.8	2683.5

Table 2. Determination of the cysteine residue content of the low molecular mass ISPIs purified from the immune hemolymph of *G. mellonella*. The ISPIs were treated with 4-vinylpyridine under reducing condition followed by reversed-phase HPLC purification and MALDI-TOF MS.

Inhibitor	Mass of untreated ISPI (Da)	Mass of alkylated ISPI (Da)	Mass shift (Da)	Mass shift per alkylated Cys (Da)	Cys residues (*n*)
ISPI-1	9223	10 490	+1267	+105	12
ISPI-2	6337	6970	+633	+105	6
ISPI-3	8269	8921	+652	+105	6

Determination of isoelectric points

The isoelectric points (pI) of the purified protease inhibitors were determined by IEF on a pH 3–10 gel (Novex) run according to the manufacturer's instructions; proteins were stained with silver.

RESULTS

Detection of inducible serine protease inhibitors within *G. mellonella* hemolymph

Trypsin inhibitory activity was detected in cell-free hemolymph of untreated *G. mellonella* larvae. This activity increased 1 h after injection of zymosan and reached maximum levels 12 h post injection (Fig. 2). The rapid increase of trypsin inhibitory activity within the first 4 h after injection at first glance suggests the release or activation of stored molecules. However, actinomycin D or cycloheximide caused a significant reduction of otherwise enhanced levels of trypsin inhibitory activity within the hemolymph when injected in sublethal concentrations simultaneously with the zymosan (Fig. 1). These results suggest that induced synthesis of secreted proteins is probably responsible for the strong increase of trypsin inhibitory activity within the hemolymph measured 8 h after injection. Untreated *G. mellonella* belonging to different developmental stages exhibited fluctuating trypsin inhibitory activity. Significantly enhanced levels were observed in pupae 1 day after pupation (Fig. 3).

Purification of inducible serine protease inhibitors from *G. mellonella* hemolymph

Preliminary experiments revealed that the trypsin inhibitory activity within the cell-free hemolymph is stable towards acid as well as heat. Therefore, a trichloroacetic acid precipitation

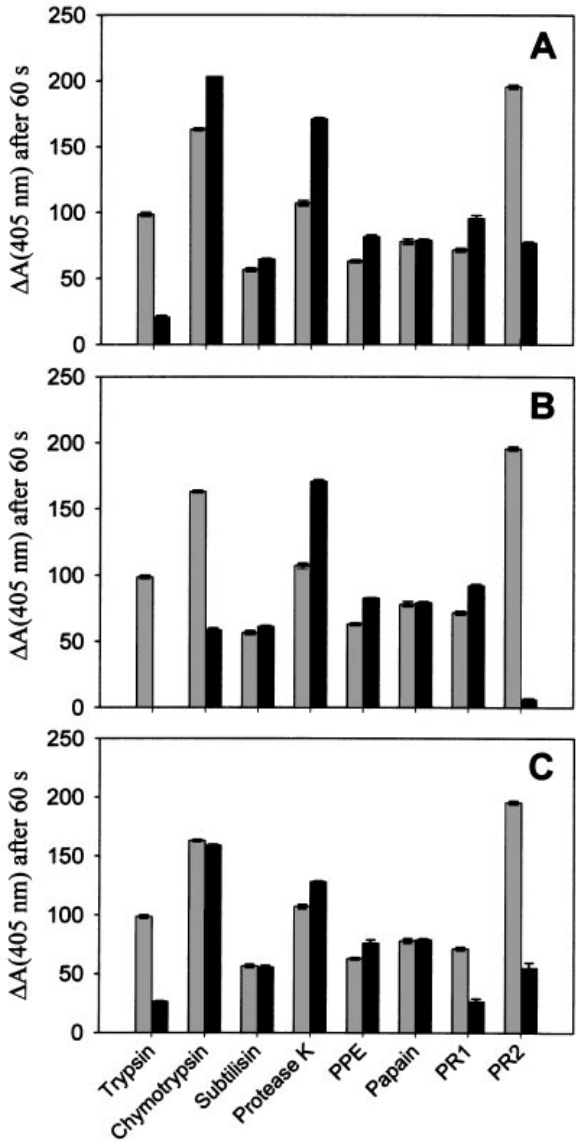

Fig. 6. Inhibitor specificity of the low-molecular-weight ISPIs. Equivalent amounts of purified ISPIs were incubated with the serine proteases bovine pancreatic trypsin, α-chymotrypsin, subtilisin *Carlsberg*, protease K, porcine pancreatic elastase, the toxic chymoelastase PR1 and trypsin-like-protease PR2 from *M. anisopliae* or the cysteine protease papain and assayed for residual proteolytic activity using chromogenic peptide P-nitroanilide substrates. Grey bars show the proteolytic activity of the proteases tested, black bars show their proteolytic activity with inhibitor added. (A) Inhibitory specificity of ISPI-1. (B) Inhibitory specifity of ISPI-2. (C) Inhibitory specifity of ISPI-3. $\Delta A_{405\ nm}$ for 1 min is displayed after incubation for 10 min at room temperature. Data are given as means ± SD of four measurements ($n = 4$).

Table 3. N-terminal amino-acid sequences of the low molecular mass ISPIs from *G. mellonella* as determined by Edman degradation. Residues in parentheses could not be unequivocally determined: in these cases, the residue is likely to be one of the two amino acids given. Amino acids marked 'x' could not be determined.

Inhibitor	N-terminal amino-acid sequence	Total amino acids sequenced
ISPI-1	DLVxGTNFxKNNPxSTRVAANSxRSPSVYRQxxxxHAKKAAxPPAVVTLL	50
ISPI-2	LDPKxTLPLETGIxRAELHRFGYDTKLKExTQFVYGGxHHNENNFKKLEVxR	52
ISPI-3	NKTNLKQLKAEAARKKAxMQDMCTSVKVEPLxAx(K/G)NGA(Y/K)RM	41

step was combined with heat treatment to prepare the starting material for further purification. Dialysis and freeze-drying served as a desalting step. After reconstitution in buffer, the proteins were separated by anion exchange chromatography on a Resource Q column. The trypsin inhibitor activity did not bind to this column and was collected in the effluent. After concentration by ultrafiltration through a 3-kDa membrane, the sample was dried by vacuum centrifugation. Material reconstituted in buffer was applied onto a Superose-12 column (Fig. 4A). A peak of trypsin inhibitory activity was detected in fractions 45–54. The fractions with highest activity were pooled and used for further analysis.

SDS/PAGE after gel filtration revealed that the resulting material is still a complex mixture composed mainly of low molecular mass components in the range 3–22 kDa. After reversed-phase HPLC separation of this material on a RP-318 column, the activity against trypsin was detected in two major and one minor peaks (Fig. 4B). The peaks with inhibitory activity were collected separately and subjected to reversed-phase HPLC again. Electrophoretic separation of the fractions obtained yielded single bands (Fig. 4C). Approximately 20 μg of purified ISPI-1 and ISPI-2 and 10 μg purified ISPI-3 were recovered from 10 mL hemolymph (Table 1).

Molecular characterization

MALDI-TOF MS revealed that the low molecular mass ISPIs contained single peaks with molecular masses of 9223 Da (ISPI-1), 6337 Da (ISPI-2) and 8269 Da (ISPI-3). Upon treatment with the alkylating reagent 4-vinylpyridine under reducing conditions, mass shifts corresponding to additions of 12 (ISPI-1) and six (ISPI-2 and ISPI-3) residues were observed while the number of mass peaks remained constant (Table 2). This observation lead to the conclusion that ISPI-1–3 consist of single chains, containing 12 or six cysteine residues, respectively. The amount of free cysteine was not determined. However, their heat-stability suggests stabilization of the molecules by disulfide bridges. No mass shift was detected upon enzymatic deglycosylation with N-glycosidase F or N-glycosidase A, suggesting that ISPI-1,2,3 do not contain N-linked carbohydrates. The isoelectric points (pI) of the inhibitors are 7.2 (ISPI-1), 7.4 (ISPI-2) and 8.3 (ISPI-3) as determined by IEF. When the ISPI1,2,3 were subjected to N-terminal sequencing by Edman degradation, strong signals provided reliable sequencing of 40–52 amino acids indicating the purity of the proteins. N-terminal sequencing of ISPI-1–3 yielded the sequences shown in Table 3. The sequences of ISPI-1 and ISPI-3 are not similar to any known proteins, whereas ISPI-2 exhibits strong similarity to the group of bovine pancreatic trypsin inhibitor-Kunitz-inhibitors (Fig. 5). The presence of an Arg residue at the putative P position is consistent with activity of ISPI-2 as an inhibitor of trypsin.

Inhibitory activity of the ISPIs

All three of the purified serine protease inhibitors are significantly active against bovine pancreatic trypsin (which was used for detection) and the toxic trypsin-like protease Pr2 from *M. anisopliae*. ISPI-2 had additional activity against α-chymotrypsin, and ISPI-3 was capable of inhibiting the toxic chymoelastase Pr1 from *M. anisopliae*. All inhibitors were inactive against the serine proteases subtilisin, protease K, porcine pancreatic elastase and the cysteine protease papain (Fig. 6).

DISCUSSION

Three inducible low molecular mass protease inhibitors have been successfully purified from cell-free hemolymph of *G. mellonella* larvae. Using actinomycin D to block transcription of genes and cycloheximide to inhibit translation they were shown to be induced during humoral immune response against the injected fungal elicitor zymosan. The soluble components of this yeast cell wall preparation have been demonstrated to induce anti-fungal humoral immune responses in *G. mellonella* [8]. Our results do not allow estimation of the extent to which the isolated inhibitors are responsible for the trypsin inhibitory activity detected within the cell-free hemolymph of untreated larvae. The relatively rapid increase of this activity in response to injected zymosan, already detectable 1 h after injection, suggests the release of stored or activated protease inhibitors within the hemolymph. This observed phenomenon can be explained, for example, by degranulation of granular cells, which are immune competent hemocytes present in the hemolymph of insects [1]. This cell type is known to contribute to cellular defense reactions and to degranulate upon contact with microbial surfaces, the exocytosed material has yet not been completely characterized but serine protease inhibitors have been identified [1]. The additional time dependent increase of trypsin inhibitory activity measured within cell-free hemolymph 8 h after injection of microbial elicitors of humoral immune responses, reaching maximum levels 12 h post injection, could be suppressed by simultaneous injection of actinomycin D and cycloheximide. This result suggests that the induced activity is at least partially due to neosynthesis of protease inhibitors.

The stability of the protease inhibitory activity against heat and trichloroacetic acid precipitation enabled efficient removal of background proteins. Subsequent FPLC and HPLC separation combined with desalting and concentrating steps led to the purification of three trypsin inhibitors which were characterized according to their molecular masses, isolelectric points and N-terminal amino-acid sequences. Screening for the substrates of the identified molecules revealed activity against Pr1 and/or Pr2, the major serine proteases produced by *M. anisopliae* during infection of host insects. Both enzymes have been considered to contribute to the virulence of this fungus used worldwide to control insect pests [2].

The occurrence of heat stable inhibitors against *M. anisopliae* Pr1 and Pr2 within the hemolymph of *G. mellonella* was discovered by Kucera [9] who partially purified and identified three heat stable serine protease inhibitors with different isoelectric points [10]. Although the results of the molecular characterization regarding the isolelectric point of inhibitors purified in our study do not correspond to the properties of the three molecules which were partially purified by Kucera, we assume that we identified the protease inhibitors which were originally discovered by him because we did not detect other serine protease inhibitors within the heat-stable fraction of hemolymph preparations. The differences concerning the isolelectric points of the determined protease inhibitors may be due to different purification procedures used in the two studies. N-terminal amino-acid sequencing of the purified inducible protease inhibitors revealed no significant similarity of ISPI-1 and ISPI-3 to other known proteins, suggesting the discovery of novel protease inhibitors, whereas the N-terminal amino-acid sequence of ISPI-2 exhibits extensive similarity to Kunitz-type protease inhibitors. Among invertebrates, members of the Kunitz family have been isolated from sea anemones and

several arthropods. Among other lepidopteran insects two trypsin and chymotrypsin inhibitors from the Kunitz family have been isolated from the hemolymph of *Manduca sexta* [29], and a family of three chymotrypsin inhibitors from the silkworm *Bombyx mori* have been characterized. ISPI-2 is most similar to *Manduca* Kunitz inhibitor B.

The demonstrated induction of the protease inhibitors in response to injected microbial elicitors of humoral immune reactions in insects and their activity against *M. anisopliae* proteases suggests their participation in anti-fungal defense reactions. As proteases produced by *M. anisopliae* have been localized on the surface of invading hyphae [4] and were demonstrated to inhibit attachment, spreading and phagocytic activity of isolated *G. mellonella* plasmatocytes, another dominant immune competent hemocyte type in insects, it is plausible that the proteases could contribute to a mechanism mediating suppression of cellular immune responses within the infected host [3]. *G. mellonella* larvae preinjected with zymosan or heat-inactivated yeast cells exhibit enhanced inhibitory activity against fungal proteases within the cell-free hemolymph and prolonged survival after subsequent infection with injected spores of virulent fungal strains [11].

The biological function of the inducible protease inhibitors purified from cell-free *G. mellonella* hemolymph is probably not restricted to the regulation of proteases released by invading pathogens. The serine protease inhibitory activity within the hemolymph of lepidopteran species studied are under the control of hormones and fluctuates during development [30]. In *G. mellonella* we observed an up-regulation during pupation, suggesting regulation of endogenous proteases involved in metamorphosis. The role of ISPI-1–3 may not be restricted to the inhibition of microbial proteases; they might also regulate endogenous proteases that are activated in response to microbial infection. Other serine protease inhibitors in invertebrates such as the serpins, high molecular serine protease inhibitors, are assumed to regulate pathways of protease cascades that ultimately activate enzymes involved in blood coagulation and melanization [31].

ACKNOWLEDGEMENTS

This research was supported by the Deutsche Forschungsgemeinschaft (DFG) Go 165/14-3/4 and NIH grant GM41247.

REFERENCES

1. Gillespie, J.P., Kanost, M.R. & Trenczek, T. (1997) Biological mediators of insect immunity. *Ann. Rev. Entomol.* **42**, 611–643.
2. Clarkson, J.M. & Charnley, A.K. (1996) New insights into the mechanisms of fungal pathogenesis in insects. *Trends Microbiol.* **4**, 197–203.
3. Griesch, J. & Vilcinskas, A. (1998) Proteases released by entomopathogenic fungi impair phagocytic activity, attachment and spreading of plasmatocytes isolated from haemolymph of the greater wax moth, *Galleria mellonella*. *Biocontr. Sci. Technol.* **8**, 517–531.
4. St. Leger, R.J., Frank, D.C., Roberts, D.W. & Staples, R.C. (1992) Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene from the entomopathogenic fungus, *Metarhizium anisopliae*. *Eur. J. Biochem.* **204**, 991–1001.
5. St. Leger, R.J., Bidochka, M.J. & Roberts, D.W. (1994) Isoforms of the cuticle-degrading Pr1 proteinase and production of a metalloproteinase by *Metarhizium anisopliae*. *Arch. Biochem. Biophys.* **313**, 1–7.
6. St. Leger, R.J., Bidochka, M.J. & Roberts, D.W. (1994) Characterization of a novel carboxypeptidase produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Arch. Biochem. Biophys.* **314**, 392–398.
7. Gupta, D.K., Leathers, T.D., El-Sayed, G.N. & Ignoffo, C.M. (1994) Relationship among enzyme activities and virulence parameters in *Beauveria bassiana* and *Trichoplusia ni*. *J. Invertebr. Pathol.* **64**, 13–17.
8. Vilcinskas, A. & Matha, V. (1997) Antimycotic activity of lysozyme and its contribution to humoral anti-fungal defence reactions of *Galleria mellonella*. *Anim. Biol.* **6**, 19–29.
9. Kucera, M. (1982) Protease inhibitor of *Galleria mellonella* acting on the toxic protease from *Metarhizium anisopliae*. *J. Invertebr. Pathol.* **38**, 33–38.
10. Kucera, M. (1984) Partial purification and properties of *Galleria mellonella* proteolytic inhibitors acting on *Metarhizium anisopliae* toxix protease. *J. Invertebr. Pathol.* **43**, 190–196.
11. Vilcinskas, A. & Wedde, M. (1997) Inhibition of *Beauveria bassiana* proteases and fungal development by inducible protease inhibitors in the haemolymph of *Galleria mellonella* larvae. *Biocontr. Sci. Technol.* **7**, 591–601.
12. Wedde, M., Weise, C., Kopacek, P., Franke, P. & Vilcinskas, A. (1998) Purification and characterization of an inducible metalloprotease inhibitor from hemolymph of greater wax moth larvae, *Galleria mellonella*. *Eur. J. Biochem.* **255**, 535–543.
13. Jiang, H. & Kanost, M.R. (1997) Characterization and functional analysis of 12 naturaly occurring reactive site variants of serpin-1 from *Manuca sexta*. *J. Biol. Chem.* **272**, 1082–1087.
14. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254.
15. Ramesh, N., Sugumaran, M. & Mole, J.E. (1988) Purification and characterization of two trypsin inhibitors from the hemolymoh of *Manduca sexta* larvae. *J. Biol. Chem.* **263**, 11523–11527.
16. Sasaki, T. (1978) Chymotrypsin inhibitors from hemolymph of the silkworm, *Bombyx mori*. *J. Biochem.* **84**, 267–276.
17. Sasaki, T. (1984) Amino acid sequence of a novel Kunitz-type chymotrypsin inhibitor from hemolymph of silkworm larvae, *Bombyx mori*. *FEBS Lett.* **168**, 227–230.
18. Sasaki, T. (1988) Amino-acid sequences of two basic chymotrypsin inhibitors from silkworm larval hemolymph. *Biol. Chem. Hoppe Seyler* **369**, 1235–1241.
19. Papayannopoulos, I. & Biemann, K. (1992) Amino acid sequence of a protease inhibitor isolated from *Sarcophaga bullata* determined by mass spectrometry. *Protein Sci.* **1**, 278–288.
20. Nakamura, T., Hirai, T., Tokunaga, F., Kawabata, S. & Iwanaga, S. (1987) Purification and amino acid sequence of Kunitz-type protease inhibitor found in the hemocytes of horseshoe crab (*Tachypleus tridentatus*). *J. Biochem.* **101**, 1297–1306.
21. Tschesche, H. & Dietl, T. (1975) The amino-acid sequence of isoinhibitor K form snails (*Helix pomatia*). A sequence determination by automated Edman degradation and mass-spectral identification of the phenylthiohydantoins. *Eur. J. Biochem.* **58**, 439–451.
22. Antuch, W., Berndt, K., Chavez, M., Delfin, J. & Wüthrich, K. (1993) The NMR solution structure of a Kunitz-type proteinase inhibitor from the sea anemone *Stichodactyla helianthus*. *Eur. J. Biochem.* **212**, 675–684.
23. Zykova, T.A., Vinokurov, L.M., Markova, L.F., Kozlovskaya, E.P. & Elyakov, G.B. (1985) Amino-acid sequence of trypsin inhibitor IV from *Radianthus macrodactylus*. *Bioorg. Khim.* **11**, 293–301.
24. Bikfalvi, A., Binder, A., Beress, L. & Wassermann, O. (1988) Isolation and blood coagulation inhibition of a new proteinase inhibitor from the sea anemone *Anemonia sulcata*. *Comp. Biochem. Physiol.* **89B**, 305–308.
25. Ritonja, A., Meloun, B. & Gubensek, F. (1983) The primary structure of *Vipera ammodytes* venom trypsin inhibitor I. *Biochim. Biophys. Acta* **748**, 429–435.
26. Hokama, Y., Iwanaga, S., Tatsuki, T. & Suzuki, T. (1976) Snake venom proteinase inhibitors. III. Isolation of five polypeptide inhibitors from the venoms of *Hemachatus haemachatus* (Ringhal's corbra) and *Naja nivea* (Cape cobra) and the complete amino acid sequences of two of them. *J. Biochem.* **79**, 559–578.
27. Joubert, F.J. & Strydom, D.J. (1978) Snake venoms. The amino-acid

sequence of trypsin inhibitor E of *Dendroaspis polylepis polylepis* (Black Mamba) venom. *Eur. J. Biochem.* **87**, 191–198.

28. Creighton, T.E. & Charles, I.G. (1987) Sequences of the genes and polypeptide precursors for two bovine protease inhibitors. *J. Mol. Biol.* **194**, 11–22.
29. Ramesh, N., Sugumaran, M. & Mole, J.E. (1988) Purification and characterization of two trypsin inhibitors from the hemolymph of *Manduca sexta* larvae. *J. Biol. Chem.* **263**, 11523–11527.
30. Eguchi, M., Matsui, Y. & Matsumoto, T. (1986) Developmental change and hormonal control of chymotrypsin inhibitors in haemolymph of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol.* **84B**, 327–332.
31. Jiang, H., Wang, Y., Huang, Y., Mulnix, A., Kadel, J., Cole, K. & Kanost, M.R. (1996) Organization of serpin gene-1 from *Manduca sexta*. *J. Biol. Chem.* **271**, 28017–28023.