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Short communication

Determination of lactose and D-galactose using thio-NAD^+ instead of NAD^+

F. Shapiro, A. Shamay, N. Silanikove*

Agricultural Research Organization, The Volcani Center, Institute of Animal Science, PO Box 6, Bet Dagan 50250, Israel

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Abstract

Replacement of NAD by thio-NAD and measurement at 405 rather than 340 nm can be used in the determination of lactose and galactose. These modifications allow microplate-readers rather than UV spectrophotometers to be used in the assays.

Keywords: Lactose; D-galactose; Enzymatic analysis; β-galactosidase; β-galactose dehydrogenase

1. Introduction

Lactose is a disaccharide mainly found in the mammary gland, milk and milk products. Galactose is a monosaccharide that can be found as a degradation product of carbohydrates such as lactose and β-galactosidic oligosaccharides in foods and biological fluids. Lactose content can be determined by means of HPLC, paper chromatography or gas chromatography. However, these techniques require either expensive chemicals or instrumentation, may lack appropriate sensitivity, and are time-consuming (Kleyn, 1985). The spectrophotometric determination of lactose by hydrolysis with β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) to β-D-galactose and D-glucose followed by determining either D-galactose or D-glucose is simple, specific and inexpensive (Kleyn, 1985). Compared to the typically high levels of glucose in cells, cell culture media or blood, the level of galactose in milk is insignificant and does not affect the measurement of lactose. Thus, the analysis of lactose by oxidizing β-D-galactose according to the following reaction was established as reference method for the determination of lactose in many foodstuffs (Kleyn, 1985):

$$\beta\text{-D-galactose} + NAD^+ \xrightarrow{\beta\text{-galactose dehydrogenase}} \text{D-galactono-}\gamma\text{-lactone} + NADH + H^+.$$

The concentration of NADH formed is directly proportional to the concentration of galactose, and, hence, to the concentration of lactose hydrolyzed by β-galactosidase. NADH is measured by the increase in absorption at 339, 334 or 365 nm (Kleyn, 1985).

This UV method is specific and accurate (Kleyn, 1985). However, because the measurements of NADH require reading in the UV range, the procedure is carried out usually in single-well spectrophotometers, which make it slower and time consuming in comparison to determination with micro-plate readers. Theoretically, NAD^+ can be replaced by thio-NAD^+, as described by the following equation, with the advantage that the accumulation of thio-NADH can be monitored by the increase in absorbance in the visible range at 405 or 415 nm (Buckwalter & Meyerhoff, 1994; Takahshi et al., 1994):

$$\beta\text{-D-galactose} + \text{thio-NAD}+ \xrightarrow{\beta\text{-galactose dehydrogenase}} \text{D-galactono-}\gamma\text{-lactone} + \text{thio-NADH} + H^+.$$

The aim of this study was to test this modification, which may allow us to determine simultaneously D-galactose concentrations in many samples by using a micro-plate reader. A similar approach was applied by Takahshi et al. (1994) to determine carnitine by carnitine dehydrogenase in the presence of thio-NAD^+.

*Corresponding author. Fax: +972-8-9475075.
E-mail address: nsilanik@agri.huji.ac.il (N. Silanikove).

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2. Materials and methods

2.1. Materials

Lactose, 3-(*N*-morphilino)-propanesulfonic acid (MOPS), and thio-NAD were purchased from Sigma Chemical Co (Rehovot, Israel). Galactose dehydrogenase-S (50 μmL^{-1} in 3.2 M ammonium sulfate, pH 6), and *β*-galactosidase (1500 μmL^{-1} in 3.2 M ammonium sulfate, pH 6) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). All other chemicals and salts used were of analytical grade. The enzymatic reaction was carried out in an Automated Microplate Reader (model Elx800, Bio-Tek Instruments, Inc., Vermont).

2.2. Reagents and standards

The following reagents and standards were prepared prior to each set of analysis:

(1) The neutralizing reagent was prepared by mixing equal volume of 4 M KOH and 1 M K_3PO_4 and diluting the mixture 100-fold with double-distilled water.
(2) Reagent A was prepared by mixing 1 mL MOPS buffer (1 M KOH solution added to 0.5 M MOPS to bring the pH to 7) with 0.5 mg thio-NAD, 10 μL 0.5 M $MgSO_4$ and 4.5 μL galactose dehydrogenase-S solution.
(3) Reagent B was prepared by mixing 1 mL reagent A with 15 μL *β*-galactosidase solution.
(4) Standards containing different concentrations of D-galactose or lactose were prepared by diluting stock solutions (10 mM of either D-galactose or lactose stored at –20°C) to yield concentrations of 5, 10, 50, 100, and 250 μM.

2.3. Milk and blank samples preparation

Milk samples were taken from eight healthy lactating goats, and from the mammary secretion of eight goats during involution. The lactose concentration during involution is dramatically lower than during lactation (Oliver & Smith, 1982; Shamay, Shapiro, Mabjeesh, & Silanikove, 2002). The samples were defatted by centrifugation at 2000*g* for 15 min at room temperature. The samples were then deprotienized by mixing 50 μL of skim milk with 500 μL of double-distilled water and 500 μL of 3% trichloroacetic acid (TCA). After 15 min the supernatant was separated by centrifugation for 30 min at 2000*g*, at 4°C, and then neutralized by mixing 50 μL of supernatant with 200 μL of the neutralizing reagent and 850 μL of double-distilled water. Blank samples were prepared by mixing 550 μL of double-distilled water with 500 μL of 3% TCA. Then, 500 μL of the mixture were mixed with 2 mL of the neutralizing reagent and 8.5 mL of double-distilled water.

2.4. Lactose recovery in milk

Known quantities of lactose were added to milk; the milk was prepared for analysis as described and the recovery of the added mass of lactose was calculated. In addition, gradual quantities of lactose were dissolved in the mammary secretion sampled from goats induced into involution to create a range of lactose concentrations between 9 (basal concentration) and 300 μM. The goats were induced into involution by treating them with casein hydrolyzate as described by Shamay et al. (2002). Mammary secretion was then milked from the

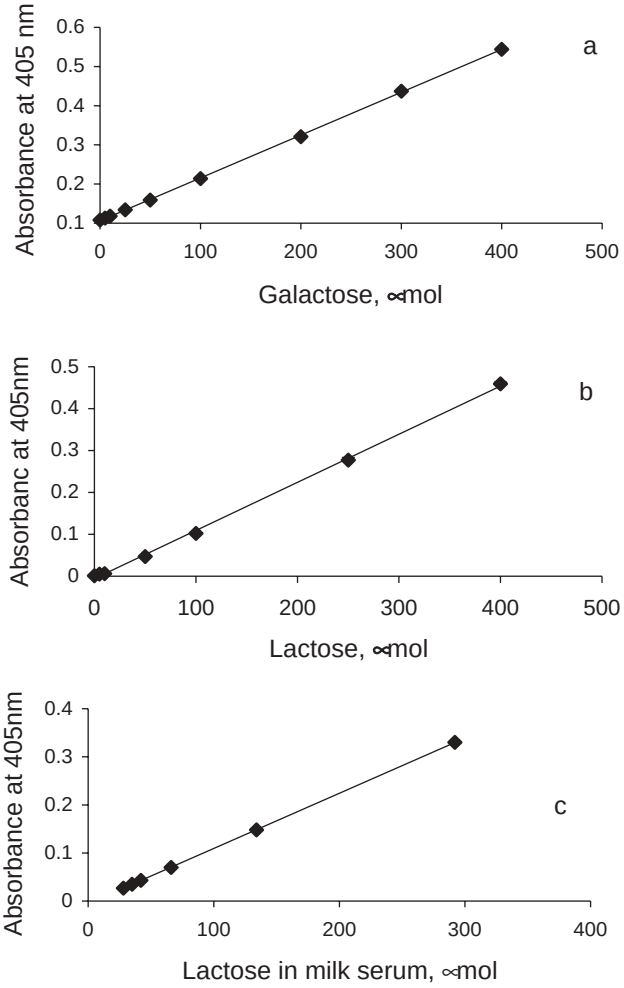

Fig. 1. Relationships between the concentrations of galactose (a), lactose (b), and lactose added to mammary secretion of goats during involution (c) and absorbency of thio-NADH. The equations of the regression lines for the relationship between absorbance and galactose concentration in water, lactose in water and lactose in mammary secretion are: Galactose (Fig. 1a): Y (absorbency) = 0.1603 + 0.0011X, $r^2 = 0.998$, Lactose (Fig. 1b): $Y = -0.0056 + 0.0011X$, $r^2 = 0.999$, Lactose added into mammary secretion of goats (Fig. 1c): $Y = -0.0054 + 0.0011X$, $r^2 = 0.999$.

Table 1
Comparison between the method of analysis: analysis of lactose concentration (mM) in goat's milk ($n = 8$) and in goat's mammary secretion during involution (MSI; $n = 8$) by the UV (NAD^+/NADH) and visible light (thio-NAD^+/thio-NADH) methods

	Method					
	UV		Visible			
	Av.	SD	Av.	SD	Difference	SD
Milk	121.1	14.52	123.3	14.32	2.2	1.22
MSI	9.42	4.002	8.71	4.602	0.7	0.63

Difference: difference between the UV and the visible methods. Analysis of the difference between the two methods by *t*-test indicate that it is not statistically different from 0 in the case of milk and MSI.

involuted glands and prepared for enzymatic assay as described above for the milk. The range of lactose concentration was chosen to be equivalent to the range of lactose concentration in the standards.

2.5. Enzymatic assays

For the D-galactose assay, 100 μL of each type of sample (i.e., milk, blank or standard) was mixed with 100 μL of reagent A in the wells of the Microplate Reader. For the determination of lactose concentration, 100 μL of sample was mixed with 100 μL of reagent B. When samples contained D-galactose and lactose, they were mixed in separate wells with reagent A in one well and reagent B in the second well. In the three procedures, the samples were incubated in the wells for 60 min at 20°C, and then the absorbance was read at 405 nm. For comparison, the samples were also analyzed by the UV method according to the Boehringer–Mannheim methodology (Kleyn, 1985). In all procedures, the samples were analyzed in triplicate.

2.6. Statistical analysis

The relationship between absorbance and galactose, or lactose concentration was analysed by standard linear regression analysis. The accuracy of the assays was based on the 95% confidence interval of the respective linear regressions.

3. Results and discussion

The highly linear relationships between galactose (Fig. 1a) or lactose (Fig. 1b) and the increase in absorbency at 405 nm indicate that the amount of thio-NADH formed is directly proportional to the amount of galactose hydrolyzed by β-galactosidase (Buckwalter & Meyerhoff, 1994; Takahshi et al., 1994). Thus, our assumption that thio-NAD can replace NAD as an oxidizing agent in β-galactosidase cleavage of galactose was proven by the present results.

The accuracy of the assay for galactose was 5 μM and that of lactose 10 μM. The recovery of lactose added to milk (12 samples) ranged between 98% and 102%. The regression between the concentration of lactose added to the mammary secretion and absorbance was essentially similar to the regression between lactose standards (in water) and absorbance (Fig. 1b vs. c). Thus, both tests indicate that the treatment procedure eliminated potential interference factors from the milk or mammary secretion.

The results obtained with the procedure based on detection of thio-NADH in the visible range were very similar to those based on detection of NADH in the UV range both in milk and in mammary secretion during involution (Table 1). Thus, the new method was found to be as simple and as accurate as the UV method. Plate readers in the UV range are much more expensive than plate readers in the visible range. Thus, the modification described in this paper enables the determination of the lactose concentration in milk with commonly available microtiter plates, which make it much more rapid (and potentially automated) in comparison to the original UV method.

References

Buckwalter, K. B., & Meyerhoff, F. F. (1994). Adapting homogenous enzyme-linked competitive binding assays to microtiter plates. *Analytical Biochemistry*, *218*(1), 14–19.

Kleyn, D. H. (1985). Determination of lactose by an enzymatic method. *Journal of Dairy Science*, *68*(10), 2791–2798.

Oliver, S. P., & Smith, K. L. (1982). Bovine mammary involution following intramammary infusion of colchicine and endotoxine at drying off. *Journal of Dairy Science*, *65*(5), 801–813.

Shamay, A., Shapiro, F., Mabjeesh, S. J., & Silanikove, N. (2002). Casein-derived phosphopeptides disrupt tight junction integrity, and precipitously dry up milk secretion in goats. *Life Sciences*, in press.

Takahshi, M., Ueda, S., Misaki, H., Sugiyama, N., Matuso, N., & Muaro, S. (1994). Carnitine determination by an enzymatic cycling method with carnitine dehydrogenase. *Clinical Chemistry*, *40*(5), 817–821.