Identification of lactose ureide, a urea derivative of lactose, in milk and milk products

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ABSTRACT

With the widespread consumption of milk, the complete characterization of the constituents of milk and milk products is important in terms of functionality and safety. In this study, a novel nonreducing carbohydrate was separated from powdered skim milk and was identified using electron spray ionization-mass spectrometry (m/z 385.1[M + H⁺]), ¹H, ¹³C, ¹H¹H-correlation spectroscopy, and heteronuclear single quantum-nuclear magnetic resonance spectra. The carbohydrate was identified as a lactose derivative of urea, *N*-carbamoyl- α - β -D-galactopyranosyl-(1-4)-D-glucopyranosylamine (lactose ureide, LU). For the HPLC analysis of LU in milk and milk products, benzoylated LU, hepta- α -benzoyl lactose ureide (melting point 137–139°C; m/z 1,113 [M + H⁺]; wavelength of maximum absorption, $\lambda_{\max}$, 229 nm; molar extinction coefficient, ϵ , 8.1037×10^4), was used as a standard. The crude nonreducing carbohydrate fraction from raw milk, thermally processed milk, and milk products such as powdered milks were directly benzoylated and subjected to HPLC analysis using an octadecylsilyl column to determine the quantity of LU. The content of LU in 10% solutions of powdered skim milk and powdered infant formula (5.0 ± 1.1 and 4.9 ± 1.5 mg/L, respectively) were almost 3-fold higher than that of UHT milk (1.6 ± 0.5 mg/L) and higher than that of low-temperature, long-time-processed (pasteurized at 65°C for 30 min) milk (1.2 ± 0.3 mg/L) and the fresh raw milk sample (0.3 ± 0.1 mg/L). A time-course of the LU content in raw milk during heating at 110°C revealed that LU increased with time. From these results, it is likely that LU is formed by the Maillard-type reaction between the lactose and urea in milk and milk products. Because the concentration of LU in milk increased with the degree of processing heat treatment, it could serve as an indicator of the thermal deterioration of milk. Although it

is known that the human intestine is unable to digest LU, the gastrointestinal bacteria in human subjects are able to digest and utilize urea nitrogen in formation of essential amino acids that are available to the host human. These findings suggest that LU in milk might have a functional role in human health.

Key words: lactose ureide, lactose, urea, milk

INTRODUCTION

Nonreducing carbohydrates comprise the most abundant group of natural compounds. Although the composition of milk has been extensively characterized, no reports have been found of the identification of a nonreducing sugar in any type of milk or associated milk product. This study aimed first to separate novel nonreducing sugars from bovine milk and determine their detailed structure. Our group previously reported the development of an effective method for the separation of nonreducing sugars (Suyama et al., 1989). This approach was applied to powdered skim milk, and the subsequent structural identifications of isolated nonreducing sugars were carried out. One unique sugar was separated from skim milk and identified as *N*-carbamoyl- α - β -D-galactopyranosyl-(1-4)-D-glucopyranosylamine, also known as lactose ureide (LU), a urea derivative of lactose. No reports were found of LU being identified in any type of milk or milk products, so qualitative and quantitative analyses of LU in various types of sterilized milks and powdered milk products were performed by HPLC.

Markedly different patterns of bacterial growth have been observed between LU and lactose when these were supplied as a carbon source during an in vitro study (Merry et al., 1982a,b). In ruminant nutrition, LU is potentially beneficial both in terms of reduction of toxicity of the NPN source and its efficiency of use for rumen microbial synthesis (Merry et al., 1982a,b,c). The results suggest that LU is a source of NPN with a built-in supply of readily available carbohydrate to ruminants such as bovine. Recent studies on humans have also shown that LU is completely resistant to the

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body's digestive process (Jackson et al., 1999). Although the action of lactase may release galactose and glucose ureide from LU (Ruemmele et al., 1997), when LU is taken orally, LU is hydrolyzed by the gastrointestinal bacteria in normal human subjects and urea nitrogen of LU can be utilized in the formation of essential amino acids that are available to the host human (Ruemmele et al., 1997). These findings suggest that LU in milk might have a functional role in human health.

It is well recognized that thermal processing changes the composition of milk (Jones et al., 1998; Cataldi et al., 1999), and the Maillard reaction is the main cause of these changes. The Maillard reaction occurs between carbonyl groups, such as those present in reducing sugars, and the amino group of proteins. In milk, lactose represents the main reducing sugar involved in this reaction and it forms various products (Albala-Hurtado et al., 1999; Ferrer et al., 2000; Siciliano et al., 2000). The compounds formed by the Maillard reaction during the processing and sterilization of milk are used as an index of the degree of heat treatment. For example, lactulose is used as an index to distinguish between various types of milks. Moreover, furosine [ϵ -*N*-(2-furoylmethyl-L-lysine)], which is generated by acid degradation of the Amadori rearrangement reaction products of the Schiff base between lactose and lysine residues of milk proteins, has also attracted attention as a potential indexing substance (Evangelisti et al., 1999; Morales and Jimenez-Perez, 2000; Ohta et al., 2002). Lactose ureide is likely formed by a Maillard-type reaction between lactose and urea, and it could serve as an indicator of the thermal deterioration of milk. As mentioned above, milk components are fundamentally important for human health. In this study, we attempted to quantify the novel nonreducing sugar LU in raw milk, powdered infant formula, skim milk, and heated milk. We also investigated the relationships between the thermal process and LU formation.

MATERIALS AND METHODS

Samples

The milk samples used in this study were 5 powdered infant formulas (commercially available from Japan; 2 lots each of A and B brands, and 1 lot of C brand), 5 different lots of skim milk powder (D brand, Japan), and 5 samples of commercially available UHT-processed (pasteurized at 120°C for 2 s) milk (2 different lots of E and F brands and 1 G brand), and 3 commercially available samples of low-temperature long-time (LTLT)-processed (pasteurized at 65°C for 30 min) milk. Three different samples of raw milk were freshly obtained from 3 healthy Holstein cows from the Tohoku

University stock farm. Heated raw milk samples were prepared by heating at 110°C for 30 min and fresh raw milk samples of fresh raw milk by heating for 1 h.

Materials

Silica Gel 60 (particle size 63–200 μ m) for column chromatography and thin-layer chromatography (TLC) plates (Silica Gel 60 precoated on a 20 $\times$ 20 cm aluminum sheet) were purchased from Merck (Darmstadt, Germany). The Sep-Pak silica cartridge column (5 mL) was purchased from Waters (Milford, MA). Deuterium oxide (99.97%) and deuterated chloroform (CDCl_3 ; 99.8% deuterium) were purchased from Euriso-Top (Gif-Sur-Yvette, France). All other chemicals were of specially prepared reagent grade and were obtained from Nacalai Tesque (Kyoto, Japan).

Instruments

Electrospray ionization mass spectrometry (ESI-MS) was performed using a Finnigan MAT TSQ700 instrument (Thermo Scientific, Franklin, MA). First-atom bombardment mass spectrometry was performed using a Jeol Mstation JMS-700 instrument (Jeol, Tokyo, Japan). For this analysis, sample in *m*-nitrobenzyl alcohol was loaded on the stainless steel target, which was then bombarded with xenon atoms. ^1H and ^{13}C -nuclear magnetic resonance (NMR) spectra were recorded in D_2O or CDCl_3 at 600 MHz with a Unity INOVA 600 spectrometer (Varian, Palo Alto, CA) operated at 50°C.

Preparation of Nonreducing Carbohydrate Fraction from Skim Milk

Skim milk powder (200 g) was dissolved in 500 mL of hot water (60°C), 1 L of methanol was immediately added to the solution, and the mixture was maintained at 4°C overnight. The resulting precipitate, which consisted predominantly of amorphous proteins and lactose crystals, was removed by centrifugation at 28,000 $\times g$ for 20 min at 4°C. The H_2O -methanol supernatant layer was concentrated using a rotary evaporator under reduced pressure to 500 mL. The lactose in the concentrate was then crystallized by cooling at 4°C for 1 wk, and lactose crystal was separated followed by centrifugation at 28,000 $\times g$ for 20 min at 4°C. The aqueous layer, which consisted predominantly of carbohydrates, was used as the crude carbohydrate fraction for subsequent purification.

Isolation of the Nonreducing Sugar Fraction

To eliminate the remaining lactose in the crude carbohydrate fraction of skim milk, an aqueous layer was

treated with aniline as described previously (Suyama et al., 1987). Briefly, the aniline reagent was prepared by mixing aniline with acetic acid at a ratio of 5:2 (vol/vol). An aqueous solution of the sample was mixed with an identical volume of aniline reagent and then heated at 85°C for 5 h. After cooling the reaction mixture to room temperature, the deep brown amorphous polymer was separated from the aqueous solution by centrifugation at $28,000 \times g$ for 20 min at 4°C. The remaining brown aqueous fraction was treated separately 3 times with diethyl ether and ethyl acetate. The nonreducing sugars and ketoses, which were shown in our previous report (Suyama et al., 1987) to be not reactive toward aniline, were thus present in the aqueous fraction. Carbohydrates were separated using silica gel column chromatography (20 mm internal diameter $\times$ 150 mm), with a solvent system of (3:2:1:1 n-butanol:acetic acid:diethyl ether:water, by vol). Each fraction of carbohydrate was collected and the solvent was evaporated with a rotary evaporator until dryness. Unidentified carbohydrate corresponding to the LU was then crystallized (prism) from methanol and underwent structural analysis.

TLC of Carbohydrates

Thin-layer chromatography was performed using n-butanol:acetic acid:diethyl ether:water (3:2:1:1, by vol) as a solvent with 2 developments. Each spot was visualized by spraying a solution containing 2.0% diphenylamine, 2.0% aniline, and 22% phosphoric acid in acetone onto the plate, which was then heated at 120°C for 5 min.

Synthesis of LU

Lactose ureide was synthesized by the method used for the synthesis of glucose ureide as described by Merry et al. (1982a). Briefly, equal weights (100 g) of lactose and urea were dissolved in 800 mL of distilled water, and then 20 mL of sulfuric acid (4.5 M) was added under stirring. The resulting reaction mixture was maintained at 70°C for 5 d with shaking for 1 min twice daily. The resultant faintly colored greenish-yellow liquid was concentrated to approximately 200 mL under reduced pressure at 37°C using a rotary evaporator. Methanol (300 mL) was added to the thin syrup obtained and the mixture was kept at 4°C for 48 h to allow crystallization to occur. The obtained crystals were separated through filter paper (Toyo No. 2, Advantec, Tokyo, Japan) using a Buchner funnel, washed in 100 mL of methanol, and then refiltered. Recrystallization was carried out using methanol:water (1:2, vol/vol) to yield 80 g of LU. The crystals were dried in vacuum desiccator over di-

phosphorus pentoxide. The compound was charged on structural analysis.

Synthesis of Hepta-*o*-Benzoyl LU

Ten grams of dried LU was added to 800 mL of benzoylation reagent [benzoic anhydride (50 mg/mL) and 4-dimethylaminopyridine (25 mg/mL) in dry pyridine], mixed, and then incubated for 24 h at 37°C. The reaction mixture was concentrated to approximately 300 mL under reduced pressure at 37°C using a rotary evaporator. Chloroform (100 mL) was then added and the resulting mixture was stirred for 10 min at room temperature. The chloroform fraction was separated and then washed with ice water, 3% HCl, and 3% NaHCO₃. The chloroform fraction was concentrated using a rotary evaporator under reduced pressure as described by Chaturvedi et al. (1997). The concentrate was charged onto a silica gel column for flash chromatography in hexane:ethyl acetate (4:1, vol/vol). hepta-*o*-benzoyl LU was collected as a syrup, and then crystallized from methanol to afford plate, with a melting point of 137 to 139°C. Nuclear magnetic resonance δ_H (CDCl₃): 4.60 (doublet, d, J = 9.1 Hz, H1), 4.24 (d, J = 7.5 Hz, H1), 3.72 (doublet of doublet, dd, J = 1.7, 10.1 Hz, H6'), 3.71 (d, J = 3.9 Hz, H4), 3.58 (dd, J = 8.2, 11.7 Hz, H6), 3.75 (dd, J = 8.0, 10.1 Hz, H6'), 3.54 (dd, J = 4.1, 11.7 Hz, H6), 3.50 (dd, J = 4.1, 8.2 Hz, H5), 3.48 (t, J = 8.9 Hz, H3'), 3.44 (dd, J = 3.3, 9.9 Hz, H3), 3.43 (dd, J = 7.7, 8.9 Hz, H4'), 3.42 (ddd, J = 1.7, 7.7, 8.0 Hz, H5'), 3.34 (dd, J = 8.0, 9.9 Hz, H2), 3.19 (t, J = 9.1 Hz, H2). Fast atom bombardment mass spectra: m/z 1,113, UV (CH₃CN): λ_{max} (ϵ): 229 nm (4.572×10^7).

Preparation of Milk Samples for HPLC Analysis

Methanol (2 mL) was added to 1 mL of each liquid milk sample and to 10% aqueous solutions of powdered infant formulas and skim milk powders. Each resulting solution was mixed by vortexing and then centrifuged at $1,500 \times g$ for 20 min. Two milliliters of supernatant was treated by adding an aniline reagent (aniline:acetic acid, 5:2, vol/vol) and then heated at 85°C for 5 h. After cooling the reaction mixture to room temperature, the solution was washed twice with 4 mL of diethyl ether. The aqueous layer was concentrated to dryness and then further dried over diphosphorus pentaoxide under reduced pressure. To each dried sample, 2 mL of benzoylation reagent [benzoic anhydride (50 mg/mL) and 4-demethylaminopyridine (25 mg/mL) in dry pyridine] was added. After mixing, the sample solutions were incubated for 24 h at 37°C, concentrated to dryness, and then dissolved in hexane:ethyl acetate (1:1,

vol/vol). The resulting solution was run through a Sep-Pak silica cartridge column that was previously wetted with acetonitrile (5 mL) and equilibrated with 5 mL of hexane:ethyl acetate (1:1, vol/vol). After washing the cartridge with 10 mL of hexane, the benzoylated derivatives were eluted with 3 mL of ethyl acetate. The eluate was dried under reduced pressure to dryness, and dissolved in 500 μ L of acetonitrile, and then equal volumes of the solution were charged on the HPLC.

HPLC Analysis of Benzoylated Derivative of Carbohydrates

The HPLC was performed with a reverse-phase system using a Mightysil RP-18 column (4.6 $\times$ 150 mm; Kanto-kagaku, Japan). The HPLC system consisted of an L-6020 pump (Hitachi, Japan), SPD-6AV UV-visible spectrophotometric detector (Shimadzu, Tokyo, Japan), and a C-R5A Chromatopac Data Station (Shimadzu). The optical density was monitored at 229 nm. An acetonitrile:water (65:35, vol/vol) solvent system at a flow rate of 1.0 mL/min was used. All chromatographic operations were performed at room temperature. Quantitative analysis of LU was performed using a standard curve generated with known concentrations of synthesized hepta-*o*-benzoyl LU.

Statistical Analysis

One-way ANOVA was used for statistical analysis. The results are presented as means $\pm$ standard deviations.

RESULTS

The carbohydrate fraction of skim milk powder was treated with aniline reagent and subjected to TLC. The TLC showed 2 main spots with retention factor (**R_f**) values of 0.45 and 0.35, respectively, as shown in Figure 1. Because each of these spots represented an individual carbohydrate, they were purified by column chromatography and subjected to further analysis. The compound having an **R_f** of 0.45 was identified as lacturose because it showed an identical **R_f** value (Figure 1) and ^1H -NMR spectrum (data not shown) to a lacturose standard.

The purified carbohydrate corresponding to the spot with **R_f** of 0.35 was analyzed by positive ion ESI-MS, which gave a molecular ion peak $[\text{M} + \text{H}]^+$ at m/z (385.1), $[\text{M} + \text{Na}]^+$ (407.0), and $[\text{M} + \text{K}]^+$ (423.1) as shown in Figure 2. Although not shown, the negative ion ESI-MS resulted in a molecular ion peak corresponding to $[\text{M} - \text{H}]^-$ at m/z (383.1) and $[\text{M} + \text{Cl}]^-$ (419.0). The purified carbohydrate was next subjected to ^1H -NMR analysis and the result is shown in Figure 3. From the

obtained spectrum, the coupling constant of H-4 signals (3.9 Hz) supported the presence of a galactose moiety due to axial-equatorial interaction between H-3 and H-4 protons, and the coupling constant of H-4' signals (8.9 Hz) supported the presence of a glucose residue due to axial-axial interaction between H-3' and H-4' protons. An α -anomeric proton was not observed in the spectrum, and a α -anomeric proton at the glucose residue observed at 4.81 ppm was highly deshielded compared with that at the glucose residue of lactose (4.65 ppm).

The ^1H and ^{13}C -NMR signals were assigned after ^1H - ^1H correlation spectroscopy (COSY), hetero-nuclear single quantum coherence (HSQC), and hetero-nuclear multiple-bond connectivity (HMBC) analysis as follows. The nonreducing sugar fraction: ^1H -NMR δ_{H} (600 MHz, D_2O): 4.81 (d, 1H, $J_{1,2}$ 9.1 Hz, H-1'), 4.45 (d, 1H,

Figure 1. Thin-layer chromatography of the nonreducing carbohydrate fraction of separated from powdered skim milk. 1 = glucose, 2 = galactose, 3 = nonreducing carbohydrate fraction, 4 = lacturose, 5 = lactose uride.

Figure 2. Positive ion electron spray ionization mass spectrometry of the compound purified from nonreducing carbohydrate fraction of skim milk powder sample.

Figure 3. ^1H -Nuclear magnetic resonance spectrum of the compound purified from nonreducing carbohydrate fraction of skim milk powder sample. The structure of lactose ureide and assignment results are shown (inset).

Figure 4. High-performance liquid chromatograms of the hepta-*o*-benzoyl lactose ureide standard and a benzoylated sample of commercial milk. The synthesized benzoylated hepta-*o*-benzoyl lactose ureide standard (bottom) was eluted from the HPLC column after 21 min at room temperature and was detected by measuring the absorbance at 229 nm. Benzoylated lactose ureide was observed in a commercial milk sample (top).

$J_{1,2}$ 7.5 Hz, H-1), 3.93 (dd, 1H, $J_{6,6}$ 10.1 Hz, $J_{5,6}$ 1.7 Hz, H-6'), 3.92 (d, 1H, $J_{3,4}$ 3.9 Hz, H-4), 3.79 (dd, 1H, $J_{6,6}$ 11.7 Hz, $J_{5,6}$ 8.2 Hz, H-6), 3.78 (dd, 1H, $J_{6,6}$ 10.1 Hz, $J_{5,6}$ 8.0 Hz, H-6'), 3.75 (dd, 1H, $J_{6,6}$ 11.7 Hz, $J_{5,6}$ 4.1 Hz, H-6), 3.71 (dd, 1H, $J_{5,6}$ 8.2 Hz, $J_{5,6}$ 4.1 Hz, H-5), 3.69 (dd, 1H, $J_{3,4}$ 8.9 Hz, $J_{2,3}$ 8.9 Hz, H-3'), 3.65 (dd, 1H, $J_{3,4}$ 3.3 Hz, H-3), 3.64 (dd, 1H, $J_{3,4}$ 8.9 Hz, $J_{4,5}$ 7.7 Hz, H-4'), 3.63 (ddd, 1H, $J_{5,6}$ 8.0 Hz, $J_{4,5}$ 7.7 Hz, $J_{5,6}$ 1.7 Hz, H-5'), 3.55 (dd, 1H, $J_{2,3}$ 9.9 Hz, $J_{1,2}$ 8.0 Hz, H-2), 3.40 (dd, 1H, $J_{1,2}$ 9.1 Hz, $J_{2,3}$ 9.1 Hz, H-2'). ^{13}C -Nuclear magnetic resonance signals were assigned after HSQC as follows: δ_{C} (600 MHz, D_2O , 50°C): 160.67 (C = O), 103.00 (C1), 80.97 (C1'), 78.38 (C4'), 76.04 (C5'), 75.42 (C5), 75.24 (C3'), 72.70 (C3), 71.81 (C2'), 71.07 (C2), 68.68 (C4), 61.08 (C6), 60.23 (C6').

Figure 4 shows the typical HPLC chromatograms of hepta-*o*-benzoyl LU standard and a sample of commercial UHT milk benzoylated using 50 mg/mL benzoic anhydride and 25 mg/mL 4-dimethylaminopyridine in pyridine. Hepta-*o*-benzoyl LU was eluted as a single peak from the HPLC column at room temperature

after 21 min and was clearly present in the milk sample. Hepta-*o*-benzoyl LU was also observed in all milk samples, including skim milk powder, powdered infant formulas, UHT milk, LTLT milk, and raw milk (Figure 5). The average LU content in each of the milk sample was determined using a standard curve, which revealed that a 10% solution of skim milk contained 5.0 ± 1.1 mg/L, which was almost 3-fold higher than that in UHT milk (1.6 ± 0.5 mg/L). The samples of LTLT pasteurized milk contained a much lower amount of LU (1.2 ± 0.3 mg/L) than that of UHT milks. A sample of raw milk had the lowest content of LU (0.3 ± 0.1 mg/L).

To examine the effect of temperature on the formation of LU, the sample of raw milk was heated at 110°C for 30 min and 1 h. As a result of this heat treatment, the amount of LU increased from 0.3 to 8.2 ± 0.9 mg/L (at 1 h) as shown in Figure 5.

DISCUSSION

In this study, the ESI-MS and NMR analysis of the nonreducing carbohydrate fraction from skim milk revealed the presence of a novel sugar in milk. The results of these analyses allowed us to assign the purified carbohydrate compound as LU, a urea derivative of lactose. To date, no reports exist of LU being found in any type of milk. From the structure determined for LU, we speculate that it was formed by a Maillard-type condensation reaction between lactose and urea in milk.

The levels of LU in milk samples showed a relationship to the severity of processing and sterilization, with the highest concentrations being found in skim milk powder. Skim milk is manufactured by the centrifugal removal of cream from fresh milk, followed by condens-

Figure 5. Lactose ureide (LU) concentration in several types of milk samples. The concentration of LU in each sample was determined using HPLC and a standard curve generated with a synthesized LU standard.

ing by heating or spray drying to obtain skim milk powder. Although UHT milk is sterilized by either indirect or direct heating at 80 to 85°C, it is further sterilized at 120 to 130°C for 2 s before packaging, whereas pasteurized (LTLT) milk is sterilized by heating for 65°C for 30 min. Such processing changes the composition of milk. The LU content in milk and milk products is likely the identical trend observed for substances used as the official index of milk quality, namely, lacturose and furosine. Therefore, the LU detected in this study represents another possible indicator of milk quality, although future research using a larger number of samples is required.

The levels of LU in milk may also be reflected in the urea content of milk (normal range: 10–14 mg/dL). Recently, MUN has been recognized as one tool to evaluate ration protein and energy status (Kauffman and St-Pierre, 2001; Jonker et al., 2002; Nousiainen et al., 2004; Wattiaux et al., 2005). The Feed Management 592 Practice Standard was adopted by the National Resources Conservation Service (NRCS, 2003) as another tool to assist with addressing resource concerns on livestock and poultry operations. Feed management can assist with reducing the import of nutrients to the farm and reduce the excretion of nutrients in manure. Milk processing plants and DHI can provide daily managers with MUN values in bulk milk and individual cow milk samples (Wattiaux et al., 2005). The MUN level is a useful tool that can allow dairy managers to monitor changes in the feeding and management of their herds (Nousiainen et al., 2004).

On the other hand, recent studies have demonstrated that LU is completely resistant to the body's digestive enzymes, although the action of lactase may release galactose and glucose ureide (Ruemmele et al., 1997) and can be used to obtain information on aspects of the handling material by the colonic microflora in normal human subjects (Jackson et al., 1999). Our results showed that the consumption of milk and milk products led to an increase in LU intake. Although it is known that the human intestine is unable to digest LU, the precise health effects of consuming LU in milk and milk products is unknown at this time.

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