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Glycoconjugate stability in human milk: glycosidase activities and sugar release

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Abstract

Many human milk glycoconjugates (glycolipids, glycoproteins, mucins, glycosaminoglycans) and oligosaccharides are biologically active, and their activity depends on the precise structure of the glycan. The sugars on the terminus of the glycan are vulnerable to cleavage by glycosidases. Because glycoconjugates incubate together with endogenous glycosidases in the breast between feedings, and in expressed milk during storage, the presence and activity of glycosidases in human milk was investigated. α-L-Fucosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, *N*-acetyl-β-hexosaminidase, β-D-glucuronidase, and neuraminidase were measured in milk samples from 4 donors by use of synthetic fluorogenic glycosides; fucosidase and hexosaminidase displayed the highest activity. The catabolic activity of the major glycosidases was confirmed by measuring the corresponding free sugars in milk. Free fucose, *N*-acetylneuraminic acid, and *N*-acetylhexosamines were measured and their identities were confirmed by high-performance liquid chromatography, gas chromatography, and gas chromatography-mass spectrometry. Incubation of samples for 16 hr at 37°C and 20°C, but not at 4°C, resulted in time-dependent increases in the amount of free fucose, *N*-acetylneuraminic acid, and *N*-acetylhexosamines, consistent with their enzymatic release by the endogenous glycosidases. Stored frozen milk had the same levels of these sugars as did samples analyzed immediately after collection, indicating that glycosidases are inactive in the frozen milk. Samples analyzed immediately after collection contained only small amounts of free sugars, suggesting that glycoconjugate degradation during the typical residence time of milk in the breast is modest.

Keywords: Human milk; Glycosidases; Fucose; *N*-acetylneuraminic acid; *N*-acetylhexosamines

1. Introduction

Human milk contains many types of oligosaccharides and glycoconjugates (glycolipids, glycoproteins, mucins, and glycosaminoglycans), some of which are biologically active and protect against microbes, viruses, and toxins [1]. These glycoconjugates and oligosaccharides are produced and modified in mammary epithelial cells; the intracellular enzymes involved in these processes are also found in human milk [2,3]. Thus, both the glycoconjugates and carbohydrate-metabolizing enzymes, primarily glycosyltranferases and glycosidases, are secreted into lactiferous ducts. The glycosyltransferases are unlikely to be active in human milk because the concentration of available substrate (high-energy sugar phosphate nucleotides) is insufficient to support anabolism. The glycosidases, on the other hand, do not depend on sugar phosphate nucleotides and may be active in hydrolyzing free sugars from oligosaccharides and glycoconjugates. Other classes of hydrolases, e.g., lipases, are known to be active in human milk and can even function in the digestive system of the infant. However, information about the presence or function of glycosidase activity in human milk is limited.

In earlier studies, we found α-L-fucosidase activity in milk from healthy donors. This activity increases over the course of lactation [4] and is expressed only in the soluble milk fraction [5]. Neuraminidase activity had been found in human milk much earlier by use of radioactively labeled natural substrate [6]. *N*-Acetylhexosaminidase activity, although one of the highest glycosidase activities in mammalian tissues and fluids, has not, to our knowledge, been observed in human milk. The goals of the present study were (1) to define the presence and levels of glycosidase activities in human milk and (2) to determine whether, and

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to what extent, these glycosidases act on endogenous milk glycoconjugates. To achieve the latter, changes in the levels of free sugars that might be released by specific endogenous glycosidase activities were measured in milk samples that were incubated at different temperatures and for different lengths of time. The amounts of these sugars present in milk before incubation would indicate the degree to which endogenous glycosidases could affect the glycoconjugates and oligosaccharides of milk while it is stored within the breast between feedings.

2. Materials and methods

2.1. Materials

Fluorogenic substrates for glycosidase activity, 4-methylumbelliferyl derivatives of α-L-fucopyranoside, β-D-galactopyranoside and α-D-galactopyranoside, β-D-glucopyranoside, β-D-glucuronide, β-D-*N*-acetylglucosaminide, and α-D-*N*-acetylneuraminic acid (NANA), were obtained from Sigma Chemical Co. (St. Louis, MO), as were monosaccharides used for standards. Tri-Sil reagent for producing trimethylsilyl derivatives of polar compounds for gas chromatography was obtained from Pierce (Rockford, IL). All other chemicals were obtained from commercial sources and were of ACS or greater purity.

2.2. Milk samples

Human milk samples were collected from four healthy donors (JR, LS, AB, and VA at 9, 4, 4, and 2 months postpartum, respectively). Milk was pumped into plastic test tubes containing sodium azide to achieve a final concentration 0.02% of this antimicrobial. The samples were snap frozen at −68°C and stored at −85°C for no more than 1 week until analysis.

2.3. Glycosidase assay

Activity of α-L-fucosidase (EC 3. 2. 1. 51), β-D-galactosidase (EC 3. 2. 1. 23), α-D-galactosidase (EC 3. 2. 1. 22), β-D-glucosidase (EC 3. 2. 1. 21), β-D-glucuronidase (EC 3. 2. 1. 56), *N*-acetyl-β-D-hexosaminidase (EC 3. 2. 1. 52), and neuraminidase (EC 3. 2. 1. 18) were assayed routinely with the appropriate fluorogenic substrates (4-methylumbelliferyl α-L-fucopyranoside, β-D-galactopyranoside, α-D-galactopyranoside, β-D-glucoside, β-D-glucuronide, β-D-*N*-acetylglucosaminide, and α-D-NANA) at pH 5.0 (fucosidase) and 4.5 (all other glycosidases) and a final substrate concentration of 1 mM [7]. The reaction mixture (100 μL) contained 20 μL of human milk (~80 to 100 μg of protein) and 80 μL of fluorogenic substrate dissolved in 0.1 to 0.2 M citrate-phosphate buffer, pH 4.5 or 5.0. After 1 hr of incubation at 37°C the reaction was stopped with 2 mL of 0.25 M glycine-KOH buffer, pH 10.4. The fluorescence of enzymatically liberated 4-methylumbelliferone was determined in an Aminco SPF-500 C spectrofluorometer by excitation at 365 nm and emission measured at 480 nm (SLM Aminco, Spectronic Unicam, Rochester, NY). The specific activity of the glycosidase was expressed as nanomoles of hydrolyzed substrate per milliliter of milk per hour. The rates of the enzyme reactions were within the linear range with respect to time and milk volume under these conditions.

2.4. Incubation and isolation of milk monosaccharides

Individual 5-mL milk samples, which had been frozen immediately upon collection from the 4 donors, were thawed and incubated at 4°C, 20°C, or 37°C for 1 to 16 hr. Control samples were kept frozen until analysis. To confirm that freezing did not affect levels of sugars in the milk, fresh samples were obtained from the same donors and analyzed immediately after expression.

Nine volumes of ethanol (final concentration 90%) were added to precipitate proteins, lactose, and high-molecular-weight oligosaccharides. After settling overnight at 4°C, a clear supernatant was obtained by centrifugation at $3{,}000 \times g$ for 1 hr. The supernatant was concentrated on a rotary evaporator to a dry white residue, which was then extracted twice with 5 mL of 90% ethanol to enrich the monosaccharide relative to lactose. The extracts were dried on a Supelco vacuum system (Bellefonte, PA), solubilized in 1 mL of water, filtered through glass wool, and used for analysis by gas-liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS).

2.5. Preparation of the milk samples for GC and GC-MS

Aliquots (200 mL) from each sample were taken in GC vials and evaporated under N_2 at 45°C. Mannitol was used as the internal standard for all samples. Samples were desiccated over P_2O_5 overnight. Tri-Sil reagent (Pierce) was added (100 μL), and samples were left at room temperature (20°C) for 30 to 40 min to produce trimethylsilyl (TMS) ether derivatives. The TMS derivatives were dried under N_2 and were dissolved in 100 μL of dry hexane; 1 μL of this solution was injected into a 30-m DB-1 capillary column in a Hewlett-Packard gas chromatograph (Model 5890A) with an HP 3396A integrator (Hewlett-Packard, Palo Alto, CA). Solutions of neutral monosaccharides of known concentrations were used as external standards [8].

2.6. GC-MS

TMS ethers of the free sugars, prepared as described above, were analyzed by ammonia chemical ionization GC-MS using a Finnigan 4500 mass spectrometer (ThermoQuest, San Jose, CA) equipped with a Hewlett-Packard 5890 GC and a Teknivent Vector/Two data system (ProLab, Madison, WI). A 30 m × 0.25 mm DB-1 (0.25-μm phase

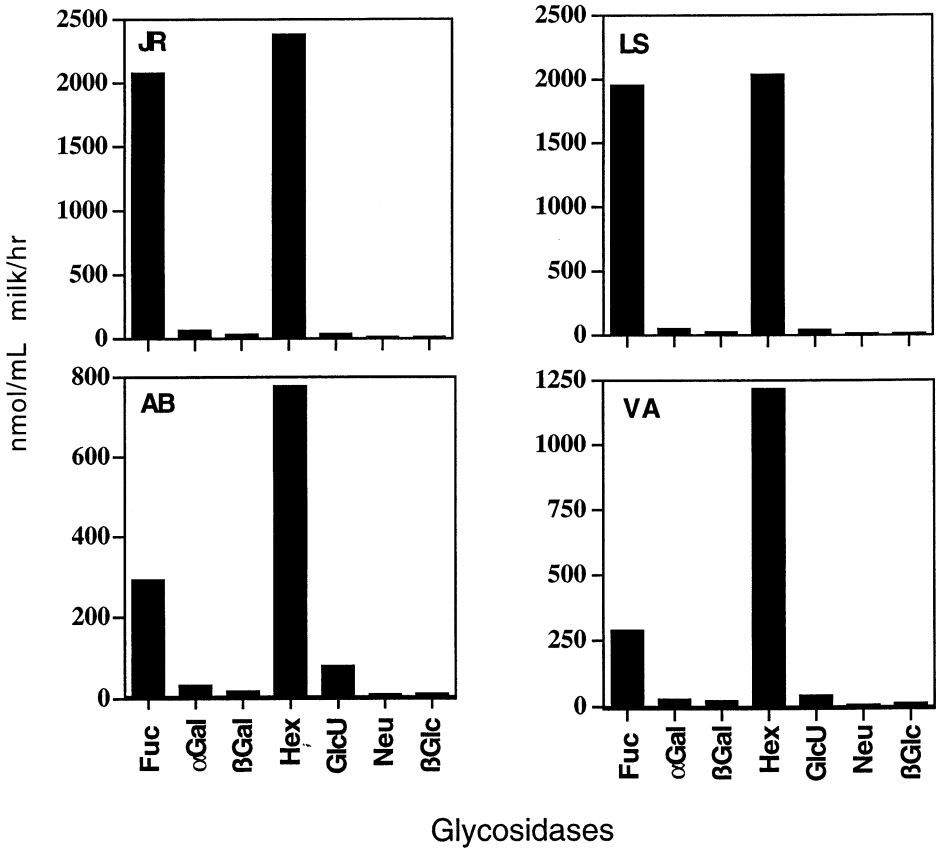

Fig. 1. Comparative glycosidase activity in whole human milk of different donors. **Fuc**, α-L-fucosidase; α**Gal**, α-D-galactosidase; β**Gal**, β-D-galactosidase; **Hex**, hexosaminidase; **GlcU**, β-D-glucuronidase; **Neu**, neuraminidase; β**Glc**, β-D-glucosidase.

thickness) fused-silica capillary column (J&W Scientific, Folsom, CA) was used with H_2 as the carrier gas at 10 psi. Direct injection was used with the injector at 320°C. The column was temperature-programmed from 100°C to 320°C at 10° per min. The ion source was maintained at 180°C with the ammonia pressure at 93 Pa. Data acquisition from m/z (mass-to-charge ratio) of 430 to 750 was initiated at 5 min (150°C) and continued through the remaining 17 min of the analysis. Peak areas for the $[M^+NH_4]^+$ and MH^+ ion plots for the neutral sugars and the MH^+ ion plots for the *N*-acetyl amino sugars were integrated and compared to external standards for the estimation of concentration of free sugars [9].

3. Results

Of the seven glycosidases measured, only two, α-L-fucosidase and *N*-acetyl-β-D-hexosaminidase, displayed appreciable activity on fluorogenic substrates (Fig. 1). The level of fucosidase in milk varied from about 250 nmol/mL/h for donors AB and VA to 2,000 nmol/mL/hr for donors JR and LS. Hexosaminidase activity showed much less variation—from about 800 (AB) to 2,000 nmol/mL/hr (JR and LS). The activities of other glycosidases were very low in the milk samples tested. Our fluorogenic assay revealed no neuraminidase activity; however, Schauer et al.[6] have demonstrated neuraminidase activity in human serum and milk using a macromolecular substrate that contained the tritium-labeled C_8-analog of *N*-acetylneuraminic acid. Our finding that NANA is in milk is consistent with neuraminidase activity. GC-MS peaks corresponding to free fucose, NANA, *N*-acetylglucosamine, and *N*-acetylgalactosamine were observed. The identity of the peaks was confirmed by comparing the peaks obtained from human milk with the GC retention times and ammonia chemical ionization mass spectra of peaks obtained with standard sugars.

The concentration of free fucose and NANA in whole human milk is shown in Fig. 2. Both sugars show great variation among individual donors. The milk samples with higher fucosidase activity (JR and LS, Fig. 1) had higher free fucose concentrations than did the other samples. The highest concentration of fucose, 36 μg/mL found in milk from JR, was approximately 10-fold higher than the 3.5 μg/mL found in milk from VA. Fig. 2 also demonstrates a reciprocal relationship between the concentrations of fucose and NANA in these donors. (It is interesting that Dische observed an inverse relationship between fucose and NANA in many mammalian glycoproteins from different tissues

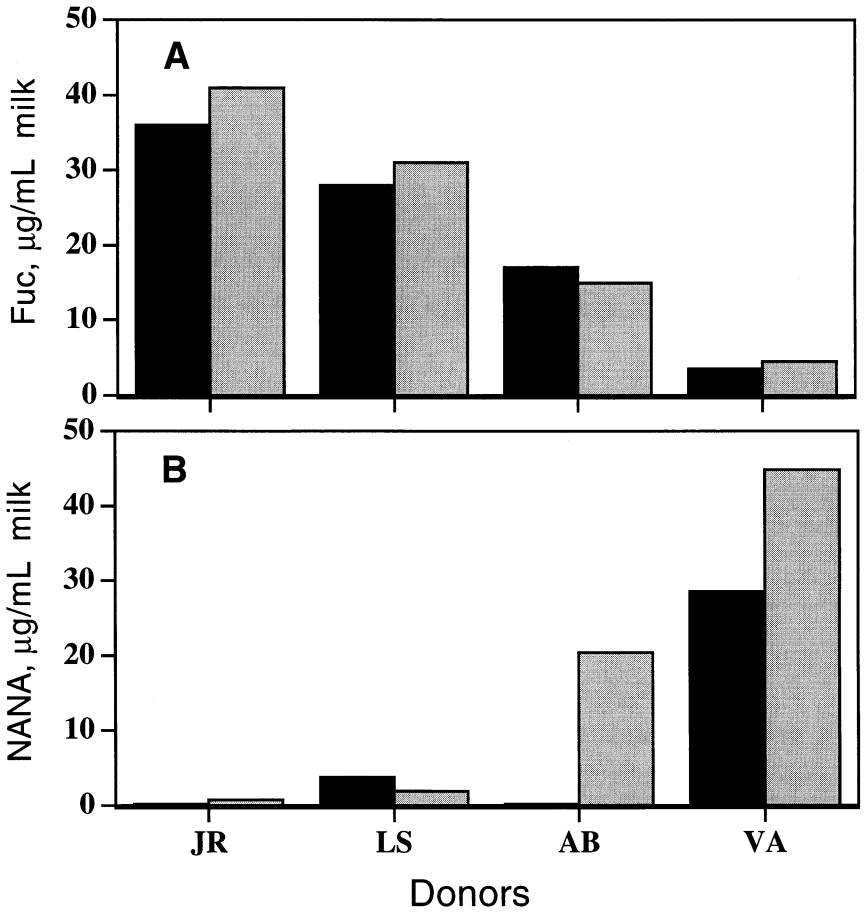

Fig. 2. The concentration of free fucose and NANA in whole human milk of different donors, determined by GC-MS. (A) Fucose; (B) NANA. Black bars, control samples kept frozen during incubation period; gray bars, samples after incubation 1 hr at 37°C.

and biological fluids [1].) Significant variability was also seen in the concentration of *N*-acetylhexosamines (*N*-acetylglucosamine and *N*-acetylgalactosamine) (Fig. 3). The highest concentrations were observed in LS, and the lowest in VA.

Each of the enzyme activities measured can cause the release of specific free sugars from glycoconjugates: fucosidase releases fucose, neuraminidase releases NANA, and hexosaminidase activity releases glucosamine and galactosamine. Therefore, to confirm the activity of these enzymes in milk, we compared free sugar concentrations in frozen milk with concentrations in samples that had been incubated for different lengths of time at various temperatures. The amount of free fucose and NANA generally increased after incubation for 1 hr at 20°C (not shown) and at 37°C (Fig. 2) but not at 4°C or frozen, consistent with the sugars' release from glycoconjugates by fucosidase and neuraminidase activities. The amount of free *N*-acetylhexosamines in milk samples from donors JR, LS, VA, and AB also increased during incubation for 1 hr at 20°C (not shown) and, with the exception of AB, at 37°C (Fig. 3) but not at 4°C or frozen. Although the levels of *N*-acetylglucosamine and *N*-acetylgalactosamine were different among these 4 donors, the relative amounts of these two sugars to one another were remarkably similar for all donors, consistent with the release of these sugars from glycoconjugates of human milk by a single hexosaminidase activity. During incubation, these free sugars in milk increased in a time-dependent manner, as illustrated by the increase in fucose levels at 37°C (Fig. 4). After 16 hours of incubation, fucose levels in milk rose over 4-fold to 170 μg/mL.

4. Discussion

The free sugars of human milk reported herein have not been described previously. The levels of the sugars found in samples kept frozen until analysis were also found in freshly expressed milk from the same donors. Thus, the sugars are not an artifact of being stored frozen or of freezing and thawing.

We had observed that human milk fucosidase demonstrates high activity at the pH of human milk and shows stability to extremes of temperature and pH [5]. Furthermore, mammalian fucosidases show activity with a wide variety of fucose-containing substrates, including synthetic

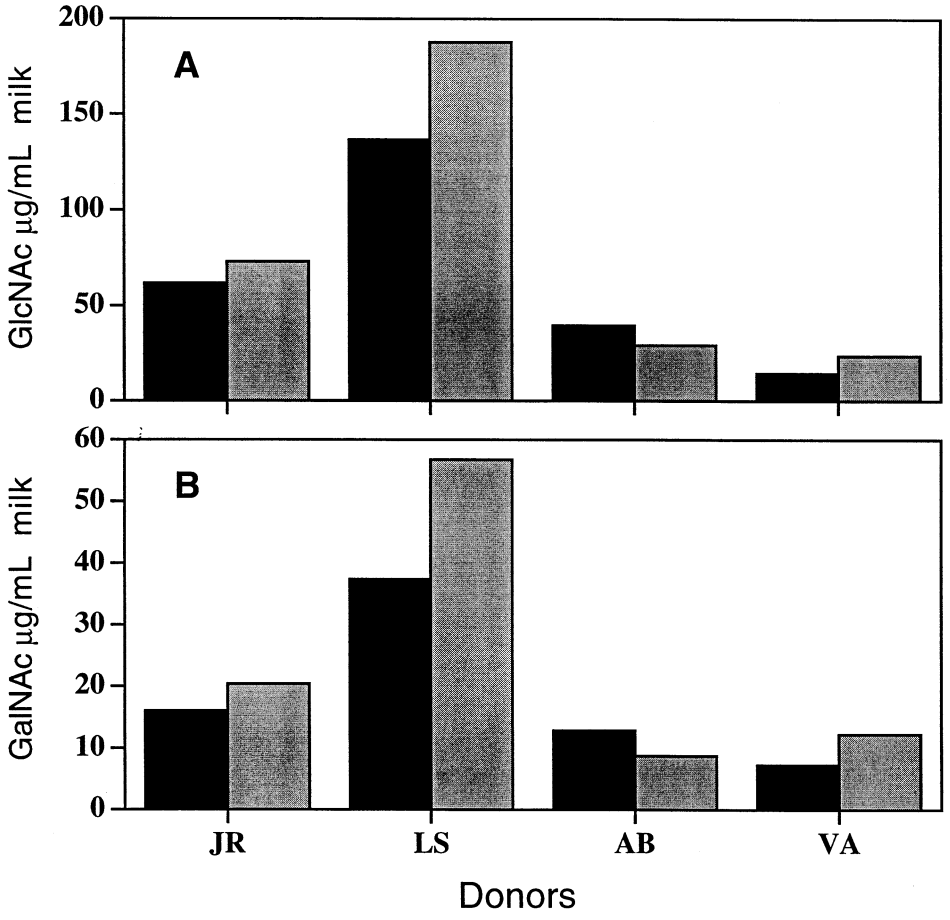

Fig. 3. Concentration of free (A) *N*-acetylglucosamine and (B) *N*-acetylgalactosamine in whole human milk of different donors. Black bars, control samples kept frozen; gray bars, samples after incubation 1 hr at 37°C.

fucose-containing glycosides and oligosaccharides [7,10], low-molecular-weight fragments of blood group A and H substances [11,12], glycopeptides, glycolipids [13–16], and milk oligosaccharides [17]. Neuraminidase activity had been observed in milk by use of an assay different from ours [6]. Hexosaminidase activity, known to be high in several fluids and tissues, had not been reported in milk.

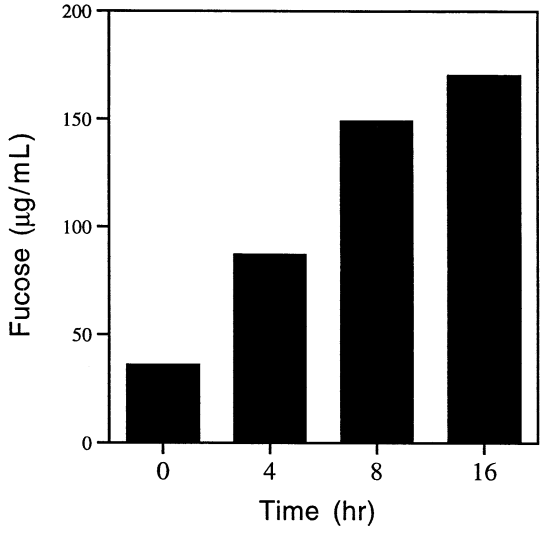

Fig. 4. Changes in fucose concentration during incubation at 37°C.

If the free fucose, *N*-acetylhexosamines, and NANA in human milk are the final products of enzymatic release from glycoconjugates and oligosaccharides, such activity would be expected to result in the temperature- and time-dependent release of these free sugars during incubation. We observed such time- and temperature-dependent increases and therefore concluded that the fucosidase, *N*-acetyl-β-D-hexosaminidase, and neuraminidase (sialidase) in human milk are capable of participating in hydrolytic modifications of the milk glycoconjugates and oligosaccharides. Freshly expressed milk also contains measurable amounts of these sugars. Between the time that milk components leave the mammary epithelial acinar cells and the milk is drawn from the breast, the milk is stored in lactiferous ducts for sufficient time for the glycosidases to release sugars from milk glycan structures. However, these sugars could also be released from surface features of the mammary cells, or could be intrinsic to proto-milk fluids as secreted by acinar cells.

However, the incremental release of free sugars during incubation indicates the maximum amount of sugar likely to be released from endogenous milk glycans by endogenous glycosidases during lactation.

Because no information is presently available concerning the specific identity of the glycoconjugates and oligosaccharides involved, or the degree to which they are hydrolyzed, the physiological significance of these glycosidases in human milk is unclear.

Despite the low levels of these free sugars, the possibility that they indicate a depletion of physiologically relevant glycoconjugates cannot be excluded at present. Possible physiological significance might be inferred by (1) comparing the levels of these sugars to those of sugars of known physiological relevance, and (2) determining the maximum amount of functional glycans that could be altered by this glycosidic activity. The most prevalent sugar in human milk is lactose (6.7×10^4 μg/mL); glucose, one of its two monosaccharide components, is present in the free form at 270 μg/mL [18]; therefore fucose, *N*-acetylglucosamine, and NANA, whose average values in this study were 21, 63, and 8 μg/mL, respectively, are present in amounts approximately one order of magnitude below glucose, indicating that their concentrations are in a range that could have biological relevance. Regarding functional glycans, some of the human milk oligosaccharides shown to have protective activity against pathogens are present in even lower concentrations than these free sugars. If a biologically active oligosaccharide or glycoconjugate were especially vulnerable to hydrolysis by the glycosidases and were to contribute disproportionately to the free sugars found in milk, the loss of that oligosaccharide or glycoconjugate could be significant.

Conversely, if biologically active and other oligosaccharides and glycoconjugates contribute more or less equally to the total amount of free sugars released, the proportion of each hydrolyzed compound would probably be biologically benign, considering the low amounts of free sugars relative to the amounts found as glycans. Even in milk samples that were incubated at normal body temperature for up to 16 hours, only 170 μg/mL of free fucose was found, approximately 5% of the available bound fucose. Thus, despite the presence of active glycosidases, storage (even at room or body temperature for limited periods) and prolonged intervals between breast feeds may modify, but are unlikely to negate, the bioactive glycoconjugates and oligosaccharides of human milk.

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References

[1] Z. Dische, Reciprocal relation between fucose and sialic acid in mammalian glycoproteins. Ann. N.Y. Acad. Sci. 106 (1963) 259–270.

[2] M. Hamosh, L.M. Freed, J.B. Jones, S.E. Berkow, J. Bitman, N.R. Mehta, B. Happ, P. Hamosh, Enzymes in human milk, in: R.G. Jensen, M.C. Neville (Eds.), Human Lactation, Plenum Press, New York, pp. 249–266, 1985.

[3] T.W. Keenan, S. Patton, The structure of milk: implications for sampling and storage, in: R.G. Jensen, (Ed.), Handbook of Milk Composition, Academic Press, Inc., San Diego, pp. 5–50, 1995.

[4] G.Y. Wiederschain, D.S. Newburg, Human milk fucosyltransferase and α-L-fucosidase activities change during the course of lactation. J. Nutr. Biochem. 6 (1995) 582–587.

[5] G.Y. Wiederschain, D.S. Newburg, Compartmentalization of fucosyltransferase and α-L-fucosidase in human milk. Biochem. Mol. Med. 58 (1996) 211–220.

[6] R. Schauer, R.W. Veh, M. Wember, Demonstration of neuraminidase activity in human blood serum and human milk using a modified, radioactively labelled α_1-glycoprotein as substrate. Hoppe-Seyler's Z. Physiol. Chem. 357 (1976) 559–566.

[7] G.Y. Wiederschain, I.K. Kozlova, G.A. Ilyina, M.A. Mikhaylova, E.M. Beyer, The use of glycosides of 6- and 8-acylamino-4-methylumbelliferone in studies of the specificity and properties of human lysosomal glycolipid hydrolases. Carbohydr. Res. 224 (1992) 255–272.

[8] J.A. Perry, Introduction to Analytical Gas Chromatography: History, Principles, and Practice. Marcel Dekker, New York 1981.

[9] R.P. Evershed, Advances in silylation. in: K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, John Wiley & Sons, Chichester, England, 1993, pp. 51–108.

[10] N.L. Bach, E.M. Beyer, G.Y. Wiederschain, N.V. Bovin, S.E. Zurabyan, A study of properties and substrate specificity of human α-L-fucosidase multiple forms using natural and synthetic fucose-containing oligosaccharides. Bioorgan. Chem. 7 (1981) 1024–1033.

[11] G.Y. Wiederschain, E.L. Rosenfeld, Specificity of pig kidney α-L-fucosidase and its action on different fragments of blood group A + H substance. Bull. Soc. Chim. Biol. 51 (1969) 1075–1084.

[12] G.Y. Wiederschain, Multiple forms of human glycosidases and their role in glycoconjugate degradation. Adv. Clin. Enzymol. 2 (1982) 150–157.

[13] R.B. Carlsen, J.G. Pierce, Purification and properties of an α-L-fucosidase from rat epididymis. J. Biol. Chem. 247 (1972) 23–32.

[14] G. Dawson, G. Tsay, Substrate specificity of human α-L-fucosidase. Archives of Biochemistry & Biophysics, 184 (1977) 12–23.

[15] R.L. Hopfer, S.W. Johnson, M. Masserini, A. Giuliani, J.A. Alhadeff, Hydrolysis of fucosyl-G_{M1} ganglioside by purified pellet-associated human brain and human liver α-L-fucosidases without activator proteins or detergents. Biochem. J. 266 (1990) 491–496.

[16] S.W. Johnson, J.A. Alhadeff, Mammalian α-L-fucosidases. Comp. Biochem. Physiol. 99B (1991) 479–488.

[17] G.Y. Wiederschain, E.L. Rosenfeld, Two forms of α-L-fucosidase from pig kidney and their action on natural oligosaccharides. Biochem. Biophys. Res. Commun. 44 (1971) 1008–1014.

[18] D.S. Newburg, S.H. Neubauer, Carbohydrates in milk, in: R.G. Jensen (Ed.), Handbook of Milk Composition, Academic Press, San Diego, pp. 273–349, 1995.