Oligosaccharides of Human Milk

STRUCTURAL STUDIES OF TWO NEW OCTASACCHARIDES, DIFUCOSYL DERIVATIVES OF PARA-LACTO-N-HEXAOSE AND PARA-LACTO-N-NEOHEXAOSE*

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Two new octasaccharides were isolated from human milk of a nonsecretor Lewis a-b- individual. Structural studies by sequential enzymic degradation and by quantitative methylation analysis revealed that their structures were as follows: \( \text{Gal} \beta_1 \rightarrow 3 (\text{Fuc} \alpha_1 \rightarrow 4 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 (\text{Fuc} \alpha_1 \rightarrow 3 \text{GlcNAc} \beta_1 \rightarrow 3 \text{Gal} \beta_1 \rightarrow 4 \text{Glc} \)).

The core portions of these sugars, which are newly found linear hexasaccharides, are named para-lacto-N-hexaose and para-lacto-N-neohexaose, respectively.

During the structural studies of the difucosyl hexasaccharide fraction of human milk, named N-3 by Kobata and Ginsburg (1), it was separated into three oligosaccharide components by prolonged paper chromatography under controlled temperature and relative humidity (2). The structure of the component with slowest mobility was studied in detail, and found to be a mixture of fucosyl lacto-N-octaose and fucosyl-lacto-N-neooctaose (2).

The other two components (N-3-1 and N-3-2) were at first thought to be mixtures of difucosyl derivatives of lacto-N-hexaose and lacto-N-neohexaose, because they gave the same monosaccharide ratio of 2 fucoses, 3 galactoses, 2 glucosamines, and 1 glucose. However, preliminary methylation studies showed that the difucosyl core portion of N-3-2 has a linear sugar chain in contrast to lacto-N-hexaose and lacto-N-neohexaose, which have branched sugar chains.

In order to find out the structure of this core oligosaccharide, a detailed study of N-3-2 fraction was performed.

RESULTS

Isolation of N-3-2 Fraction—An oligosaccharide fraction from human milk of a nonsecretor Le(a-b-) individual was obtained as previously reported (3). The fraction was further fractionated into 8 groups (1, 2, 7 and lactose) by passage through a column of Bio-Gel P-4 (Fig. 1).

This chromatographic system was useful for the effective separation of N-3-2 fraction from sialyl oligosaccharides and fucosyl lacto-N-(and neo)octaoses, because anionic materials were eluted at the void volume, and N-acetylglucosamine residue behaves as 2 mol hexose units. Fraction 5 which contained lacto-N-hexaose, lacto-N-neohexaose, monofucosyl hexaoses, N-3-1, and N-3-2 was pooled, concentrated, and mounted on Whatman No. 3MM papers as a band so that 5 mg of the sample was loaded within 1 cm. The papers were then subjected to paper chromatography with Solvent II for 4 days to obtain the N-3 fraction (1). When this N-3 fraction was rechromatographed with Solvent II for 9 days at 30° under the relative humidity of 70 to 80%, it was separated into two oligosaccharide components (N-3-1 and N-3-2). These two components were eluted separately from papers and rechromatographed with Solvent II (Fig. 2). N-3-1 was a mixture of the isomers of difucosyl derivatives of lacto-N-hexaose and lacto-N-neohexaose as already reported (1). Monosaccharide ratio of N-3-2 was determined as 2 fucoses, 3 galactoses, and 2 glucosamines for each glucose by the radio-paper electrophoretic methods (4). The content of the N-3-2 fraction in 1 liter of human milk was 17.2 mg.

Structure of Core Hexasaccharide of N-3-2—From the monosaccharide ratio, N-3-2 was supposed to be difucosyl derivatives of hexasaccharides. In order to determine the structure of their core saccharide, fucoses were removed by acid hydrolysis. Five hundred micrograms of N-3-2 fraction was hydrolyzed with 500 μg of NaBH₄, (6 x 10⁶ cpm/μmol) and 2 x 10⁴ cpm of radioactive octaitol (N-3-2₀) was obtained. This octaitol was heated in 0.01 N HCl at 100° for 1.5 h. Fucoses were mostly removed from the N-3-2₀ by this treatment. The difucosyl N-3-2₀, which behaved as a hexitol was purified by paper chromatography with Solvent II. It was then permethylated, hydrolyzed, reduced, and acetylated. Partially O-methylated sugar alcohol acetates thus obtained were analyzed with gas chromatograph-mass spectrometer. The total ion chromatogram is shown in Fig. 3B.
1,2,3,5,6-Penta-O-methyl-4-mono-O-acetyl sorbitol, 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl galactitol, 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl galactitol, 3,6-di-O-methyl-1,4,5-tri-O-acetyl-2-N-methylacetamido-2-deoxyglucitol, and 4,6-di-O-methyl-1,3,5-tri-O-acetyl-2-N-methylacetamido-2-deoxyglucitol were detected in the molar ratio of 0.9:1.0:2.0:1.3:0.7. Absence of any disubstituted monosaccharide indicated that the core hexasaccharide has a linear structure.

Monosaccharide sequence and anomic configuration of this linear core hexasaccharide were determined by enzymic degradation. When the radioactive hexaitol (1 × 10⁶ cpm) obtained from N-3-2, which was reduced with NaBH₄, of higher specific activity (2 × 10⁴ cpm/nmol), was treated with 1.5 units of jack bean β-galactosidase, a pentaitol was obtained (Fig. 4B). When this pentaitol was incubated with jack bean β-N-acetylhexosaminidase, it was converted to a tetraitol having the same mobility as lacto-N-neotetraitol and lacto-N-tetraitol (Fig. 4C). This tetraitol was further degraded to tritol by β-galactosidase digestion. The tritol was then converted to lactitol by β-N-acetylhexosaminidase digestion. These results together with the data of methylation analysis showed that the structure of the linear core hexasaccharide is as follows:

Galβ1 → 3GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1

Therefore, the core portion of N-3-2 is a novel hexasaccharide, so far not reported.

**Linkage of Fucosyl Residue**—In order to determine the location of fucosyl residues, 500 µg of N-3-2 was subjected to methylation analysis (Fig. 3A), and the data were compared with that of core hexasaccharides (Fig. 3B).

From N-3-2, 1.9 mol of 2,3,4,6-tri-O-methyl-1,5-di-O-acetyl-fucitol and 2 mol of 6-mono-O-methyl-1,3,4,5-tetra-O-acetyl-2-N-methylacetamido-2-deoxyglucitol (Fig. 3A, Peaks b and e) were detected, while 1.3 mol of 3,6-di-O-methyl-1,4,5-tri-O-acetyl-2-N-methylacetamido-2-deoxyglucitol and 0.7 mol of 4,6-di-O-methyl-1,3,5-tri-O-acetyl-2-N-methylacetamido-2-deoxyglucitol (Fig. 3A, Peaks i and j) disappeared. These data indicated that 2 fucosyl residues in the N-3-2 are located either at the C-3 or C-4 position of two N-acetylgalactosamine residues of the core hexasaccharide as follows:

\[
\text{Fucα1} \downarrow \text{Fucα1} \\
\text{Galβ1} \rightarrow 3\text{GlcNAcβ1} \rightarrow 3\text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 3\text{Galβ1} \rightarrow 4\text{Glcβ1}
\]

**Almond Emulsin α-L-Fucosidase Digestion of N-3-2**— α-L-Fucosidase purified from almond emulsin hydrolyzes fucosyl linkages of both lacto-N-fucopentitol II which has Fucα1 → 4GlcNAc grouping and lacto-N-fucopentitol III which has Fucα1 → 3GlcNAc grouping (Chart 1) (6). This enzyme, however, cannot cleave Fucα1 → 3GlcNAc linkages of monofucosyl lacto-N-octitol and monofucosyllacto-N-neooctitol (Chart 1).³

When N-3-2 with lower specific activity (500 µg, 5 × 10⁶ cpm) was digested with 0.8 millimoles of the fucosidase in 100 µl of 0.15 M citrate buffer, pH 5.0 at 37°C for 20 h, it was complementarily converted to a heptaitol releasing 1 out of 2 mol of fucosyl residues. This heptaitol was then degraded to a pentaitol by sequential digestion with β-galactosidase (3 units) and β-N-acetyhexosaminidase (1.2 units). The remaining fucose in the pentaitol can be removed by almond emulsin α-fucosidase digestion (0.9 milliunit), releasing a radioactive tetraitol. Methylation study of this tetraitol (Fig. 5) showed that this tetraitol was lacto-N-neotetraitol and not even a minor amount of lacto-N-tetraitol was included. Therefore, the fucosyl residue is linked at only the C-3 position of the proximal N-acetylgalactosamine residue of the core hexasaccharide.

**Structures of Oligosaccharides in N-3-2 Fraction**—Summarizing all the data so far described, N-3-2 fraction is elucidated to be a mixture of the two oligosaccharides A and B in Fig. 6. The ratio of A to B in the N-3-2 fraction studied was estimated as 7 to 3 by comparing the area of 4,6-di-O-methyl- and 3,6-di-O-methyl-2-N-methylacetamido-2-deoxyglucitols in Fig. 3D.

**DISCUSSION**

Oligosaccharides of human milk can be classified into seven groups by the structures of their core oligosaccharides. These core saccharides are lactose, lacto-N-tetraose, lacto-N-neotetraose, lacto-N-hexaose, lacto-N-neohexaose (3), lacto-N-octaose, and lacto-N-neooctaose (2). The two octasaccharides reported in this paper do not fall into these groups but are made up from two new core hexasaccharides. These core hexasaccharides will be named "para-lacto-N-hexaose" and "para-lacto-N-neohexaose" since they are the isomers of lacto-N-hexaose and lacto-N-neohexaose, respectively.

As the paper chromatographic mobilities of these hexasaccharides in several different solvent systems are similar to those of lacto-N-hexaose and lacto-N-neohexaose, it was impossible to confirm the occurrence of fucose free para-lacto-N-hexaose and para-lacto-N-neohexaose in milk directly.

Therefore, detailed methylation study of the hexasaccharide fraction of milk from a nonsecretor, 1a(α1) individual was performed.

Not even a trace amount of 2,4,6-tri-O-methyl-1,3,5-tri-O-acetylgalactitol was detected. This result indicated that para-lacto-N-hexaose and para-lacto-N-neohexaose occur only as fucosyl derivatives in human milk. The absence of core oligosaccharide was also found in the case of lacto-N-octaose derivatives and lacto-N-neooctaose derivatives (2). Occurrence of Fucα1 → 3GlcNAc as a sole fucosyl linkage in fucosyllacto-N-octaose and fucosyllacto-N-neooctaose was discussed as a curious phenomena, and it was interpreted as a key point in the biosynthetic pathway of these nonsasaccharides (Fig. 7). The sugar grouping Galβ1 → 3 or 4GlcNAcβ1 → 4Glcβ1 → 3GlcNAcβ1... also occurs in both fucosyl derivatives of para-lacto-N-hexaose and para-lacto-N-neohexaose. This evidence may be a strong support for the possible biosyn-

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thetic pathway in Fig. 7 including an N-acetylglucosaminyl-transferase which catalyzes the following reaction.

\[
\text{Gal} \beta_1 \rightarrow 4\text{GlcNAc} \ldots + \text{UDPGlcNAc} \rightarrow \\
\uparrow \\
\text{Fucal}
\]
\[
\text{GlcNAc} \beta_1 \rightarrow 3\text{Gal} \beta_1 \rightarrow 4\text{GlcNAc} \ldots \\
\uparrow \\
\text{Fucal}
\]

With this enzyme, lacto-N-fucopentaose III may work as a common precursor of fucosyl para-lacto-N-hexaose and fucosyl para-lacto-N-neohexaose.

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