Structural determination by negative-ion MALDI-QIT-TOFMS\(^n\) after pyrene derivatization of variously fucosylated oligosaccharides with branched decaose cores from human milk

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We prepared neutral oligosaccharide fraction from milk (blood type A, Le\(^+\)) by anion-exchange column chromatography after the removal of lipids and proteins. Further fractionation was performed by means of \textit{Aleuria aurantia} lectin-Sepharose column chromatography and reverse-phase HPLC after labeling with a pyrene derivative. This pyrene labeling allowed identification by negative-MALDI-TOFMS\(^n\) analysis of 22 oligosaccharides with decaose cores, among which 21 had novel structures. Negative ions could not be produced from neutral oligosaccharides without labeling on MALDI. Mono-, di-, tri-, and tetrafucosylated decaose fractions contained three, nine, six, and four isomers, respectively. Our method enables easy determination of fucosylated structures on the N-acetyllactosamine branches of these isomers. On negative-MALDI-TOFMS\(^n\) the fragment ions included several A and D ions, from which fucosylation on the branches could be elucidated. Other characteristic ions were also detected. Y-type cleavage at the reducing side of \(\text{-3GlcNAc}\) indicated the occurrence of type 1 chain. Specific fragment ions were produced from H, Le\(^1\), and Le\(^3\) antigens. Linkage-specific exoglycosidase digestion confirmed the structures. The results indicate that the diversity of the oligosaccharides is due to combinations of type 1 H, Le\(^1\), Le\(^3\), and Le\(^3\)/Le\(^1\) on branched decaose cores. In typical oligosaccharides, 6-branches always consist of type 2 chain, while 3-branches, such as \(\beta\) and \(\gamma\) chains, are fucosylated type 1 chains. From the viewpoint of biosynthesis, the presence of fucosylation and type 1 chain may halt elongation of the N-acetyllactosamine and promote formation of branched structures.

**Keywords:** fucosylated oligosaccharides/human milk oligosaccharides/MALDI-MS\(^n\)/negative ion/pyrene-labeling

Introduction

Human milk is known to contain oligosaccharides with larger size (from lactose to those larger than octadecasaccharides) and greater diversity as compared to bovine milk, which mainly contains small oligosaccharides, such as lactose and sialyllactose. Human milk oligosaccharides are derived from various core oligosaccharides by sialylation via \(\alpha 2\)-3 or \(\alpha 2\)-6 linkages and fucosylation via \(\alpha 1\)-2, \(\alpha 1\)-3 or \(\alpha 1\)-4 linkages. There is considerable evidence that virulent enteric bacteria and viruses initiate infection by binding to particular sugar chains of glycolipids and glycoproteins on the surface of their target cells (Sharon 1996). Due to their structural mimicry of the sugar chains of glycoproteins on the mucous membrane, human milk oligosaccharides are considered to protect breast-fed infants against infections by blocking the adhesion of pathogens (Newburg et al. 2005; Bode 2006). Therefore, milk oligosaccharides are expected to be inhibitors of infection by these bacteria and viruses (Kobata 2003).

The structures of many human milk oligosaccharides, which have cores smaller than octaose, have been identified in detail (Kobata et al. 1978; Haeuw-Fievre et al. 1993). These oligosaccharides often express ABO blood group antigens and Le antigens containing fucose. Lebilla’s group detected 58 oligosaccharides in pooled human milk, and 44 of these were fucosylated. Seven of the 10 most abundant oligosaccharides were fucosylated, accounting for approximately 46% of the entire quantity (Nimomuevo et al. 2006). Recently, various kinds of mass spectrometry have been applied to elucidate the structures of milk oligosaccharides. Negative-ion electrospray mass spectrometry with collision-induced dissociation (CID) was proved to be useful for determination of the structures of variously fucosylated oligosaccharides, and underivatized neutral oligosaccharides were identified even from mixtures on the basis of the specific fragmentation behavior of deprotonated molecules (Pfenninger et al. 2002). Many linkage-specific fragment ions were obtained from linear and branched oligosaccharides using closed-ring chromatophore labeling (Cheng and Her 2002). Chai et al. investigated various neutral oligosaccharides, including a monofucosylated lacto-N-decaose, by combined use of electrospray MS/MS and NMR spectroscopy (Chai et al. 2001, 2002, 2005; Kogelberg et al. 2004). They identified linkages by using GC-MS analysis of partially methylated alditol acetates and NMR spectroscopy. Both kinds of analysis require substantial amounts of samples. Instead, we applied linkage-specific enzyme digestion, e.g., with \(\alpha 2\)-fucosidase and \(\beta 4\)-galactosidase (followed by \(\beta 4\)-galactosyltransferase) to pyrene-labeled oligosaccharides, and measured MS of the reaction mixtures. Sub-picomole amounts are sufficient for this method. By using a combination of enzymatic modifications and MALDI-MS\(^n\), we succeeded in identifying the structures of individual isomers in mixtures of isomeric oligosaccharides which could not be originally separated by HPLC because the mass numbers of the isomers became different from each other.
The combination of pyrene labeling and negative-ion MALDI-QIT-TOF MS that we have established is a powerful tool for structural determination of neutral oligosaccharides because negative ions are not easily produced from neutral oligosaccharides in MALDI-MS. The key advantages of this method are high sensitivity and protection against loss of fucose. Furthermore, many fragment ions such as A-, D-, Y-, and C-type ions are observed, and determination of isomeric and branched structures is possible (see Amano et al. 2009). In the present study, we focused on oligosaccharides with a decaose core, the structures of which have not previously been well elucidated. Using our method, we were able to obtain detailed structures of variously fucosylated oligosaccharides from human milk.

Results and discussion

Fractionation of oligosaccharides

A neutral oligosaccharide fraction, N-2, was considered to contain mainly decaose cores because fraction N-3 contained oligosaccharides with hexaose cores and octaose cores. The oligosaccharides in fraction N-2 were labeled with pyrene butanolic acid hydrazide (PBH) and subjected to Aleuria aurantia lectin (AAL)-Sepharose column chromatography to afford three fractions: AAL-1, AAL-2, and AAL-3, which were eluted with fucose. The percent molar ratios of oligosaccharides in fraction N-2 were labeled with pyrene butanolic acid hydrazide (PBH) and subjected to Aleuria aurantia lectin (AAL)-Sepharose column chromatography to afford three fractions: AAL-1, AAL-2, and AAL-3 were 18.1, 62.5, and 19.4, respectively. It seems that most of these oligosaccharides are fucosylated and many of them contain more than two fucose residues because at least two fucose residues within an oligosaccharide are necessary for interaction with AAL at room temperature (Amano et al. 1985; Yamashita et al. 1985). Each fraction was further subjected to reversed-phase HPLC with a C18 column to obtain several fractions. Measurement of [M−H]− ions on MALDI-QIT-TOFMS of these fractions revealed that they contained various kinds of oligosaccharides, mostly showing m/z 2088 (decaose core), m/z 2234, m/z 2380, m/z 2526, and m/z 2672, corresponding to a decaose core with 0–4 fucose residues. The remaining oligosaccharides were larger species and oligosaccharides with octaose cores. We focused on further analysis of oligosaccharides with a decaose core and 0–4 fucose residues. Monomer, di-, tri-, and tetrafucosylated decaoses were named D01, D02, D03, and D04, respectively. Finally, we found three isoforms of D01, nine isoforms of D02, six isoforms of D03, and four isoforms of D04. The AAL-1 fraction contained a nonfucosylated decaose and oligosaccharides named D01-3, D02-8, and D02-9. The AAL-2 fraction contained oligosaccharides named D01-1, D01-2, D02-1, D02-2, D02-7, D03-5, D03-6, D04-3, and D04-4. The AAL-3 fraction contained oligosaccharides named D02-3, D02-4, D02-5, D02-6, D03-1, D03-2, D03-3, D03-4, D04-1, and D04-2. Interestingly, AAL did not discriminate oligosaccharides only by the numbers of fucose residues because di-, tri-, and tetrafucosylated oligosaccharides existed in both AAL-2 and AAL-3 fractions.

Determination of branched structures

Characteristic fragment ions for branched structures are produced by negative-ion CID. The oligosaccharides used in this study generated several kinds of A ions and D ions (according to the nomenclature established by Domon and Costello (1988)) in MS2. First, D4β-3 (C3/Z4β) and D2β-5 (C5/Z2α) ions demonstrated that these oligosaccharides have doubly branched structures (Figure 1). The mass values of these D ions of the oligosaccharides are indicated in Table I. The presence of two galactose residues with 3,6-linkages was also indicated by detection of 0.3A3 and 0.4A3 ions and 0.3A5 or 0.4A5 ions. These 0.3A and 0.4A ions contain 6-linked branches, and the D ions result from Z-cleavage of the 3-linkage and C-cleavage of 3,6-linked galactose (Cheng and Her 2002; Chai et al. 2002, 2005). Unlike electrospray MS/MS of nonlabeled oligosaccharides, Yα, Yδ, and Yγ ions (Figure 1) were also produced from pyrene-labeled oligosaccharides by the release of branching antenna on MALDI-MS and were useful for structure determination.

The decaose core structure so far reported is doubly branched with a type 2 chain on the α chain, and type 1 chains on the β and γ chains (Chai et al. 2005). In addition to this core, we found a novel decaose core with type 2 chains on the α and β chains and a type 1 chain on the γ chain (see below). Linear decaose cores were not found in these fractions, but should occur in other fraction(s) which contain larger oligosaccharides, if they exist, because para-lacto-N-hexaose eluted faster than lacto-N-hexaose on HPLC.

Fucosylation on α, β, and γ chains

First, we determined the numbers of fucose residues on the α, β, and γ chains of the branched decaose core of each oligosaccharide. The total number of fucose residues can be estimated from the [M−H]− ion. 0.2A, 0.2A-H2O, and 2.4A ions at -4GlcNAc are characteristic for nonfucosylated type 2 N-acetyllactosamine (Cheng and Her 2002; Chai et al. 2002, 2005). The 2.4A4 ions and the D4β-3 (C3/Z4β) ions (Figure 1) give useful information on the occurrence of fucose in MS2 analysis of the [M−H]− ion. The D4β-3 ion indicates the number of fucoses on the α chain. For example, the D4β-3 ion at m/z 672 shows one fucose residue on the α chain. In addition, the 2.4A4 ion shows the number of fucose residues on both the α and β chains. The 2.4A4 ions at m/z 951, 1097, 1243, and 1390 are evidence for 0, 1, 2, and 3 fucose residues in total on the α and β chains, respectively. Thus, a combination of the 2.4A4 ion and the D4β-3 ion clearly shows the number of fucose residues on the β chain. Finally, any remaining fucose residue exists on the γ chain. The 2.4A4 ions observed for 22 oligosaccharides on negative-MS2 analysis are summarized in Table I. The numbers of fucose residues released by α2-fucosidase digestion are also shown in Table I.

Since β-galactosidase does not work on fucosylated N-acetyllactosamine branches, the numbers of galactose residues released by β-galactosidase represent the numbers of...
Table I. Structural information for oligosaccharides identified in this study

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<th>D ion</th>
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<th>β4Gal</th>
<th>α2Fuc</th>
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<th>γ</th>
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<td>0</td>
<td>Le⁵</td>
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</tr>
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<td>H (type 1)</td>
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</table>

Note: AAL refers to the fraction in which the oligosaccharide was contained.

Non-fucosylated chains. In Table I, the numbers of galactose residues released by β-(3,4,6)-galactosidase and β4-galactosidase are also indicated.

The above results demonstrate the extent of fucosylation on the α, β, and γ chains. For example, difucosylated D02-1 and D02-2 produced the D4β−3 ion at m/z 672 and the 2β4-ion at m/z 1097 on MS². From this D02-1 and D02-2 have one fucose on the α chain, no fucose on the β chain, and one fucose on the γ chain. The release of one galactose from D02-1 by β-(3,4,6)-galactosidase, but not β4-galactosidase, demonstrates that the β chain is Galβ1-3GlcNAc. On the other hand, D02-2 has Galβ1-4GlcNAc on the β chain because one galactose was released by β4-galactosidase. In another example, tetrafucosylated D03-1, D03-2, D03-3, and D03-4 gave the D4β−3 ion at m/z 672 and the 2β4-ion at m/z 1097 on MS². From this D03-1 and D03-2 have one fucose on the α chain, two fucoses on the β chain, and one fucose on the γ chain because D03-4 produced the 2β4-ion at m/z 1390 and the D4β−3 ion at m/z 672.

Next, fucosylated structures on α and β chains were determined by MS³ analysis of the D4β−3 ion and the 2β4-ion from each oligosaccharide. As already demonstrated in Amano et al. 2009, characteristic CID fragments were found in fucosylated oligosaccharides, such as H, L, Le⁴, and Le⁵ structures. For example, the Z ion is obtained by loss of Fucα1-2Gal (326 Da) from oligosaccharides with the H structure. Oligosaccharides with the Le⁴ structure generate the Z/Z ion (-344Da) by releasing one mole each of fucose and galactose. The ions with the Le⁵ structure produce the C/Z (D) fragment ion at m/z 364. All the D4β−3 ions at m/z 672 obtained from 15 oligosaccharides (Table I) produced the fragment ion at m/z 364 on MS³ analysis (data not shown), and this revealed that these oligosaccharides contain the Le⁵ structure on the α chain.

The 2β4-ion at m/z 1097 from various oligosaccharides were subjected to MS³ analysis and four types of mass spectra were obtained. Ion 1097a was obtained from D01-1, D02-1, and D02-4. The oligosaccharides produced the D4β−3 ion at m/z 672 at the same time on MS². The C/Z fragment ion at m/z 364 besides the Y ion at m/z 586 and the B ion at m/z 510 were detected in the MS³ spectrum, and confirmed that this ion has the Le⁵ structure (Figure 2B). The presence of the Y ion at m/z 732 indicated a nonfucosylated Galβ1-3GlcNAc residue. In the cases of D01-2 and D02-2, which also produced both the D4β−3 ion at m/z 672 and the 2β4-ion at m/z 1097 on MS², however, the fragment ions at m/z 364, 510, and 586, but not 732, were detected on MS³ of the ion at m/z 1097 (data not shown). The Y ion produced by cleavage at the reducing side of 4GlcNAc was never observed. The 2β4-ion at m/z 1103 (1097 + 6), instead of m/z 1097, was obtained after regalactosylation with [13C]galactose (Figure 3B). These results make it clear that the ion has an Le⁵ structure on the α chain and a nonfucosylated Galβ1-4GlcNAc residue on the β chain (named 1097a').

A third ion type 1097b from D01-3, as shown in Figure 2B, with the absence of the Y ion at m/z 732 suggests that this oligosaccharide ion has a nonfucosylated Galβ1-4GlcNAc residue. This is supported by the observation that the 2β4-ion at m/z 1103 was obtained after regalactosylation with [13C]galactose (Figure 3B). Because the Z ion at m/z 771 (1097–326), which is characteristic of the H structure, and the Y ion at m/z 586 were also found, the other branch has a Fucα1-2Galβ1-3GlcNAc residue.
Fig. 2. Negative-ion MS$^3$ spectra of the ions at $m/z$ 1097 as precursors. The main fragment ions are illustrated. (A) D01-1; (B) D01-3; (C) D02-3.

Fig. 3. Negative-ion MS$^2$ spectra of the ions at $m/z$ 2234 or $m/z$ 2340 from monofucosylated decaoses as precursors. (A) D01-1; (B) [$^{13}$C]galactose-labeled D01-2; (C) [$^{13}$C]galactose-labeled D01-3.
The result of α2-fucosidase digestion also supports this structure. A fourth ion type 1097c was seen in the cases of D02-3 and D02-7. The absence of the Y ion at m/z 592 (586 + 2C) and occurrence of the $^{2,4}A_4$ ion at m/z 1103 after regalactosylation with $^{[13C]}$galactose (Figure 4A and B) indicate that the original ion has a nonfucosylated Galβ1-4GlcNAc residue. The Z/Z ion at m/z 753 (1097–344) was obtained and this suggests that the A ion has an Lea structure.

The $^{2,4}A_4$ ions at m/z 1243 containing two fucose residues from various oligosaccharides were subjected to MS3 analysis and three types of mass spectra were obtained. The first ion type 1243a from D02-5, D03-3, D03-4, and D04-2, which also produced the D$_{48-5}$ ion at m/z 672 on MS2 analysis, gave the Y ion at m/z 732, the Z/Z ion at m/z 899 (1243–344), and the C/Z ion at m/z 364 as fragment ions, as shown in Figure 5A. The original ion has an Lex structure and an Leβ structure on the α chain and an Leβ/Ley structure on the β chain. It might be inferred from the exclusive existence of type 1 H, but not type 2 H, in oligosaccharides found in this study that the Leβ structure is present, but not the Leε structure.

On MS3 analysis with the $^{2,4}A_4$ ion at m/z 1390 containing three fucose residues from D03-6 and D04-3 as a precursor ion, the Y ions at m/z 732 and m/z 878, the B ions at m/z 510 and m/z 656, and the C/Z ion at m/z 364 were detected, as shown in Figure 5D. These data suggest an Leβ structure on the α chain and an Leβ/Leε structure on the β chain.

The fucosylated structures on the α and β chains were established by the above results and are summarized in Table I. The following sections present detailed structural determination of each oligosaccharide.

**Monofucosylated decaoses: D01-1, D01-2, and D01-3**

The structures of D01-1 and D01-2 in the mixture were determined without isolation. The mixture of monofucosylated decaoses (m/z 2234) was digested with β4-galactosidase and the negative-ion MS was obtained. One galactose residue was released and converted into the ion at m/z 2072 from more than a half of the oligosaccharides, although further β-(3,4,6)-galactosidase treatment generated a single oligosaccharide ion at m/z 1910 by releasing two galactose residues (data not shown). These results mean that this fraction contained two kinds of oligosaccharides, one with two Galβ1-3GlcNAc residues (D01-2) and the other with both a Galβ1-4GlcNAc residue and a Galβ1-3GlcNAc residue (D01-1). The reaction mixture after β4-galactosidase digestion was incubated with β4-galactosyltransferase and UDP-$^{[13C]}$Gal. D01-2 provided the ion at m/z 2240 (2234 + 6) after being labeled with one $^{[13C]}$Gal and could be discriminated from the other isomer ion at m/z 2234. D01-1 and $^{[13C]}$Gal-labeled D01-2 were analyzed on negative-MS2 by selecting the ion at m/z 2234 or m/z 2240, separately. The D$_{48-5}$ ion at m/z 672 and the $^{2,4}A_4$ ion at m/z 1097 from D01-1 were detected (Figure 3A). In contrast, the D$_{48-5}$ ion at m/z 672 and the $^{2,4}A_4$ ion at m/z 1103 (1097 + 6) that
Fig. 5. Negative-ion MS3 spectra of the ions at m/z 1243 (A–C) and m/z 1390 (D) as precursors. The main fragment ions are illustrated. (A) D02-5; (B) D02-6; (C) D03-5; (D) D03-6.

contained [13C]Gal were found in the case of [13C]Gal-labeled D01-2 (Figure 3B). As mentioned in the previous section, from MS3 analysis of the ions at m/z 672 and m/z 1097 or m/z 1103, D01-1 and D01-2 have an Le^4 structure in the α chain. Since the β chain of [13C]Gal-D01-2 is a Galβ1-4GlcNAc residue, the Yβ ion at m/z 1868, the Yα/Yβ ion at m/z 1358, and the Yβ/Yγ ion at m/z 1504 were not detected (Figure 3B). The observation of the Yγ ion at m/z 1874 (1868 + 6) indicates the presence of Galβ1-3GlcNAc on the γ chain of D01-2. These observations lead to the structures of the two isomeric oligosaccharides shown in Figure 6. D01-1 is identical in structure to the oligosaccharide reported by Chai et al. (2005).

D01-3, after regalactosylation with [13C]galactose, produced the 2,4A4 ion at m/z 1103 (1097 + 6), but not the D4β−3 ion at m/z 672 (Figure 3C), and this indicated the presence of Galβ1-4GlcNAc on the α chain and a fucosylated N-acetyllactosamine residue on the β chain. Taking these results together with the results of α2-fucosidase digestion and MS3 analysis of the ion at m/z 1097, an H structure existed in the β chain (Table I). Furthermore, the Yβ fragment ion at m/z 1728 (1722 + 6) and the Yβ/Yγ fragment ion at m/z 1364 (1358 + 6) were obtained, and this shows that the β chain has type 1H structure, Fuc01-2Galβ1-3GlcNAc, as shown in Figure 6. The localization of Galβ1-4GlcNAc was confirmed by the lack of the Yα fragment ion at m/z 1868, the Yα/Yβ ion at m/z 1358, and the Yα/Yγ ion at m/z 1504 from [13C]Gal-D01-3. Because the Yγ ion at m/z 1874 (1868 + 6) was detected, the γ chain is Galβ1-3GlcNAc, as shown in Figure 6.
Two important advantages of this approach for structural determination are apparent from these investigations. One is that the oligosaccharide isomers can be easily discriminated by negative-ion MS$^n$ after pyrene labeling. Second, even in the case of a mixture of isomeric oligosaccharides like D01-1 and D01-2, specific enzymatic treatments can afford oligosaccharides with different mass numbers, and the resulting oligosaccharides can be separately subjected to MS$^n$ analysis without isolation from the mixture.

**Difucosylated decaoses: D02-1, D02-2, and D02-4**

Difucosylated decaoses D02-1 and D02-2 have Le$^a$ on the $\alpha$ chain, as shown in Table I. The [M–H]$^-$ ion at m/z 2386 (2380 + 6), obtained after labeling of D02-2 with $^{13}$C$\text{Gal}$, was subjected to MS$^2$ analysis, and the D ion at m/z 672 and the $^3A_4$ ion at m/z 1103 (1097 + 6) were observed (Figure 7B). These results indicated that D02-2 has Galβ1-4GlcNAc on the $\beta$ chain, while D02-1 has Galβ1-3GlcNAc on the $\beta$ chain because galactosidase did not modify the oligosaccharide (Table I). These structures are also consistent with the appearance of the Y$\beta$ ion at m/z 2015 and the Y$\alpha$/Y$\beta$ or Y$\beta$/Y$\gamma$ ion at m/z 1504 (Figure 7C). On MS$^3$ analysis by selecting the ion at m/z 2015 from D02-1 and D02-4, the Z ion at m/z 1689 (2015–326) produced by the release of Fuc-Gal was obtained from D02-1 (Figures 8A and 9A), but not from D02-4, which has no $\alpha$2-fucosyl linkage (Table I). In contrast, the Z/Z ion at m/z 1671 (2015–344) produced by the release of Fuc and Gal was obtained from D02-4, but not D02-1. The defucosylated oligosaccharide ion at m/z 2088 was obtained from D02-4 by the release of two fucose residues after $\alpha$3/4-fucosidase digestion produced the fragment ion at m/z 1358 via the release of the $\beta$ and $\gamma$ chains. Therefore, the $\beta$ and $\gamma$ chains have Galβ1-3GlcNAc, and an Le$^a$ structure exists on the $\gamma$ chain. The structures of D02-1, 2, and 4 are illustrated in Figure 10.

**Difucosylated decaoses: D02-5 and D02-6**

As indicated in Table I, the results of negative-MS$^2$ and MS$^3$ analysis and $\alpha$2-fucosidase digestion indicated that the difucosylated decaose D02-5 has the Le$^a$ structure on the $\alpha$ chain and the Le$^a$ structure on the $\beta$ chain, while D02-6 has the Le$^a$ structure on the $\alpha$ chain and the H structure on the $\beta$ chain. Because
no galactose was released by β4-galactosidase digestion (Table I) and the Yγ ion at m/z 2015 and the Yα/Yγ or Yβ/Yγ ion at m/z 1504 were obtained on negative-MS² of the ion at m/z 2380 (Figures 8D and 9D), both isomers have Galβ1-3GlcNAc on the γ chain. In negative-MS³ of D02-5, the Z/Z ions were produced by the release of Fuc and Gal on selecting the ion at m/z 2015 (Figures 8B and 9B), as well as that at m/z 1243 (Figure 5A). This is consistent with Leα structure in D02-5. On the other hand, on negative-MS³ analysis selecting the ion at m/z 2015 or m/z 1243 from D02-6, the Z ion at m/z 1689 (Figures 8C and 9C) or m/z 917 (Figure 5B) was obtained by the release of Fuc-Gal. The Yα/Yβ ion at m/z 992 was found in the
Difucosylated decaoses: D02-3 and D02-7
As shown in Table I, the other difucosylated isomers D02-3 and D02-7 have the Leα structure on the β chain. One galactose was released by β4-galactosidase digestion (Table I). The \( \text{2,4} \_\text{A4} \) ion at m/z 1103 (1097 + 6), but not the \( \text{Dgal} \_\text{-}3 \) ion at m/z 672, was obtained from both oligosaccharides after labeling with \([^{13}\text{C}]\text{Gal}\) on negative-MS\(_2\) selecting the ion at m/z 2386 (2380 + 6) (Figure 4A and B). In addition, the Yα ion at m/z 2015 and the Yα/Yβ or Yα/Yγ ion at m/z 1504 were not detected on MS\(^3\). These results indicate the presence of Galβ1-4GlcNAc and two fucose residues were released by 2-fucosidase digestion. The difucosylated oligosaccharide produced the fragment ion at m/z 1358 by releasing β and γ chains due to the presence of type 1 chain. D02-3 has the Leα structure on the β and γ chains (Figure 10).

On MS\(^2\) analysis of \([^{13}\text{C}]\text{Gal-D02-7}\) (Figure 4B), the Z fragment ion at m/z 2060 (2386–326) and the Z/Z ion at m/z 2042 (2386–344) were observed and this indicates the presence of Leα and H structures. The monofucosylated oligosaccharide ion at m/z 2234 after α2-fucosidase digestion was subjected to MS\(^2\) analysis, and the Yγ ion at m/z 1869 and the Yβ/Yγ ion at m/z 1358 were obtained. MS\(^2\) analysis of the monofucosylated oligosaccharide ion at m/z 2234 after α3/4-fucosidase digestion showed the Yβ ion at m/z 1869 and the Yβ/Yγ ion at m/z 1358. Therefore, the β and γ chains have type 1 structure (Figure 10).

Difucosylated decaoses: D02-8 and D02-9
The difucosylated oligosaccharide fraction that did not interact well with immobilized AAL was a mixture of isomers D02-8 and D02-9. The \([^{13}\text{C}]\text{Gal}\) labeled oligosaccharides produced two kinds of \( \text{2,4} \_\text{A4} \) ions at m/z 957 (951 + 6) and m/z 1249 (1243 + 6) on MS\(^2\) analysis (Figure 4C). The \( \text{2,4} \_\text{A4} \) ion at m/z 1243 obtained without \([^{13}\text{C}]\) label showed the same MS\(^3\) pattern as 1243c in Figure 5C, and this indicates the presence of the Leβ or Leγ structure on the β chain (Table I). The other isomer, which produced the \( \text{2,4} \_\text{A4} \) ion at m/z 957, has the Leβ or Leγ structure on the γ chain. These oligosaccharides have one mol each of Galβ1-3GlcNAc and Galβ1-4GlcNAc on two chains because one galactose was released by β4-galactosidase digestion and two galactoses were released by β-3,4,6-galactosidase digestion (Table I). The Yβ/Yγ ion at m/z 1364 (1358 + 6), but not the Yα/Yβ or Yα/Yγ ion at m/z 1358 or m/z 1649, was seen on MS\(^2\) analysis of the ion at m/z 2386 (2380 + 6) obtained from \([^{13}\text{C}]\text{Gal}\) labeled oligosaccharides. These results confirm the presence of Galβ1-4GlcNAc on the α chain and Galβ1-3GlcNAc on the β or γ chain (Figure 10). The MS\(^3\) spectrum obtained by selecting the ion at m/z 2015 shows the Y fragment ion at m/z 1358, generated by the release of the Leβ/Leγ structure (Figures 8D and 9D).

Trifucosylated decaoses: D03-1, D03-2, D03-3, and D03-4
These four trifucosylated isomers were resistant to β-galactosidase (Table I), and only the ion at m/z 2015 among Yα, Yβ, and Yγ ions was detected on MS\(^2\) at m/z 2526 (Figure 11A–C). From these results, all the branches were considered to be fucosylated.
the β and γ chains (Figure 12). The Z ions at m/z 2200 and m/z 1689 were found in the MS² fragments of D03-2 (Figure 11B).

MS³ analysis of the 2,4 A₄ ion from D03-3 and D03-4 showed that the isomers have the Le⁺ structure on the α chain and the Le⁰ structure on the β chain (Table I). D03-3 did not release any fucose residues upon α2-fucosidase digestion, but three fucose residues were released in the case of α3/4-fucosidase digestion. The defucosylated oligosaccharide produced the fragment ion at m/z 1358 by the release of the β and γ chains on MS³ analysis (data not shown), and this shows that D03-3 has the Le⁺ structure on both the β and γ chains (Figure 12). D03-4 has an α2-fucosyl residue on the γ chain. After α2-fucosidase digestion of D03-4, further analysis showed that the resulting defucosylated oligosaccharide was identical with D02-5. Therefore, the structure of D03-4 is as shown in Figure 12.

**Trifucosylated decaose: D03-5**

D03-5 has a Galβ1-4GlcNAc residue on the α or β chain because the 2,4 A₄ ion at m/z 1249 (1243 + 6) was produced on MS² of the ion at m/z 2532 (2526 + 6) after [13C]Gal labeling (Figure 11D). The B ion at m/z 656 suggested the presence of the Le⁰ or Le⁺ structure. MS³ analysis of the A ion at m/z 1243 confirmed that D03-5 has the Le⁰ or Le⁺ structure on the β chain (Table I). The results of α2-fucosidase digestion and the appearance of the Yγ ion at m/z 2021 (2015 + 6) on MS² of the [13C]Gal-labeled oligosaccharide indicated the presence of type 1H structure on the γ chain. The difucosylated oligosaccharide obtained by α2-fucosidase digestion was identical with D02-8 on MS² analysis (data not shown). Therefore, the structure of D03-5 is as shown in Figure 12.

**Trifucosylated decaose: D03-6**

MS³ analysis of the 2,4 A₄ ion at m/z 1390 indicated that D03-6 has the Le⁺ structure on the α chain and the Le⁰/Le⁺ structure on the β chain. D03-6 has Galβ1-3GlcNAc on the γ chain because no galactose was released upon β4-galactosidase digestion, and the Yγ ion at m/z 2161, the Yα/Yγ ion at m/z 1650, and the Yβ/Yγ ion at m/z 1504 were seen in the MS² spectrum (Figure 11E). The structure is shown in Figure 12.

**Tetrafucosylated decaoses: D04-1 and D04-2**

The MS spectrum showed that the tetrafucosylated decaose isoforms in the AAL-3 fraction were divided into two kinds of oligosaccharides after α2-fucosidase digestion. One fucose was released from D04-1 and no fucose was released from D04-2. On MS² analysis by selecting the ion at m/z 2526 (de-α2fucosylated D04-1) after α2-fucosidase digestion, the 2,4 A₄ ion at m/z 1097 and the D₄α-3 ion at m/z 672 were obtained, while the 2,4 A₄ ion at m/z 1243 was observed before the release of the α2-fucosyl residue. In addition, the appearance of the Yβ ion at m/z 2161 in the MS² spectrum of de-α2fucosylated D04-1 reveals that D04-1 has type 1H structure on the β chain. The remaining two fucose residues should exist on the γ chain, and detection of the B ion at m/z 656 and the Yγ ion at m/z 2015 is consistent with this (data not shown). The suggested structure is shown in Figure 13.

On MS² analysis by selecting the ion at m/z 2672 (D04-2) after α2-fucosidase digestion, the 2,4 A₄ ion at m/z 1243 and the D ion at m/z 672 were obtained (Figure 14A). On MS³ analysis, the 2,4 A₄ ion was identified as 1243a (Table I). Therefore, the Le⁺
structure, Leα structure, and Leβ or Leγ structure were present on the α, β, and γ chains, respectively (Figure 13).

Tetrafucosylated decaoses: D04-3 and D04-4

The other tetrafucosylated decaose isomers in the AAL-2 fraction also contain two kinds of oligosaccharides. D04-3 has one α2-fucosyl residue and was resistant to β-galactosidase (Table I). In contrast, D04-4 has one Galβ1-4GlcNAc residue and no H structure (Table I). The mixture of isomers was labeled with [13C]Gal to generate different ions, and MS² analysis was done by selecting the ion at m/z 2672 for D04-3 or the ion at m/z 2678 (2672 + 6) for D04-4. From the ion at m/z 2672 of D04-3, the 2,4-A4 ion at m/z 1390 and the Dγ−3 ion at m/z 672 were obtained (Figure 14B), and further MS² analysis revealed the structure indicated in Table I. The trifucosylated oligosaccharide obtained by α2-fucosidase digestion was analyzed with MS² and proved to be identical to D03-6. Therefore, the Leα structure, Leβ or Leγ structure, and type 1H structure were present on the α, β, and γ chains, respectively (Figure 13).

On MS² analysis by selecting the ion at m/z 2678 (2672 + 6) of D04-4 after labeling with [13C]Gal, the 2,4-A4 ion at m/z 1249 (1243 + 6), the B ion at m/z 656, and the Yβ and Yγ ion at m/z 2021 (2015 + 6) were detected (Figure 14C). The 2,4-A4 ion was identified as 1243c (Table I) and the D (C6/Z2) ion at m/z 1554 was identical with that of D03-5 (Figure 11D). From these results, the α chain has Galβ1-4GlcNAc, and the Leβ or Leγ structure exists on both the β and γ chains. The structure is illustrated in Figure 13.

Finally, 22 fucosylated oligosaccharides including 21 novel structures were identified using the milk from a person (blood group A, Leβ positive). This diversity arises from a variety of

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**Fig. 12.** Structures of D03-1, D03-2, D03-3, D03-4, D03-5, and D03-6. The main fragment ions are illustrated.

**Fig. 13.** Structures of D04-1, D04-2, D04-3, and D04-4. The main fragment ions are illustrated.
Fig. 14. Negative-ion MS$^2$ spectra of the ions at $m/z$ 2672 or $m/z$ 2678 from tetrafucosylated decaoses as precursors. (A) D04-2; (B) D04-3; (C) $^{13}$C-galactose-labeled D04-4.

Fig. 15. Possible biosynthetic pathways of oligosaccharides. (A) Relationships among the oligosaccharides found in this study showing putative involvement of fucosyltransferases; (B) possible biosynthetic route to a branched dodecaose core from a tetraose core.
combinations of blood group antigens H, Le^a, Le^b, and Le^b or Le^b. This structural diversity seems likely to be related to the biological functions of these molecules, which presumably act through ligand mimicry of glycoproteins and glycolipids on the surface of intestinal epithelium. That is, milk oligosaccharides may be involved in various carbohydrate–protein interactions. Indeed, the isomers show different binding characteristics to AAL, used in this study. It is known that affinity for AAL increases in the order of type 1 H, Le^a, Le^b, and Le^b or Le^b (Yamashita et al. 1985). In this study, oligosaccharides with the Le^a or Le^a structure rather than type 1H structure interacted more strongly with AAL, while the occurrence of the Le^b or Le^b structure appeared to reduce the interaction. One possible reason for this is that the pyrene derivative at the reducing terminal may interfere with the binding between AAL and Le^b/Le^b structure on the β or γ chain.

We found many kinds of oligosaccharides with branched cores and polyfucosylation in the milk from the individual used in this study. It is considered that these polyfucosylated oligosaccharide structures are synthesized by combination of two kinds of fucosyltransferases which are expressed in the individual, and possible pathways are shown in Figure 15A, taking account of the fact that α2-fucosyltransferase does not act on Le^a and Le^a structures. It should be noted that oligosaccharides with a decaose core may not be always be the precursors because elongation and fucosylation occur competitively. In other words, the linear addition of N-acetyllactosamine usually occurs on galactose of Galβ1-4GlcNAc, but not on galactose that is fucosylated or linked to galactose of Galβ1-4GlcNAc at the C3 position (Galβ1-3GlcNAc) in normal cells, although extension of type 1 chains has been reported in some cancer cells (Stroud et al. 1992). This would generate the branched structure. In human milk, type 1 chains are more common than type 2 chains. For example, as shown in Figure 15B, lacto-N-tetraose or fucosylated tetraoses would be converted into lacto-N-hexaose or fucosylated hexaoses, then iso-lacto-N-octaose or fucosylated octaoses, and further, oligosaccharides with branched decaose. Furthermore, we have evidence that fucosylated dodecaoses with two N-acetyllactosamine units on the α chain are formed in this milk, as shown in Figure 15B.

Material and methods

Preparation of oligosaccharides from human milk

The oligosaccharides containing decaose cores were isolated from human milk obtained from a healthy volunteer (blood group A, Le^b positive). Fats and proteins were removed by extraction with chloroform:methanol:water (3:2:1). Oligosaccharides were separated from lactose on a Sephadex G-25 column (5 × 67 cm) by elution with water. The neutral oligosaccharides were obtained by anion-exchange chromatography with a Super Q-Toyopearl 650M column (1.6 × 10 cm) with water as the eluent, and then acidic oligosaccharides were eluted with 0.1–0.5 M pyridinium acetate. The neutral oligosaccharides were further separated into five fractions (N-1, N-2, N-3, N-4, and N-5) by Sephadex G-25 column chromatography. The oligosaccharides in the N-2 fraction that contains oligosaccharides with decaose cores were labeled with 1-pyrene butanoic acid hydrazide as described in Amano et al. 2009. Pyrene-labeled oligosaccharides were separated into three fractions by AAL-column chromatography using 50 mM ammonium acetate, pH 7.4, and then 5 mM fucose in 50 mM ammonium acetate, pH 7.4. Further separation was done by reverse-phase HPLC on an Inertsil WP300 C18 (4.6 × 500 mm) with 25% acetonitrile as the eluent.

Enzyme reactions

Glycosidase digestion was done at 37°C overnight as follows: β-(3,4,6)-galactosidase from Streptococcus pneumoniae and Xanthomonas sp. (Sigma, 16 mU) in the provided reaction buffer, pH 5; β4-galactosidase from S. pneumoniae (Calbiochem, 0.24 mU) in the 50 mM sodium acetate buffer, pH 5; α3/4-fucosidase from Streptomyces sp. 142 (Takara Bio, 2 μU) in the 0.2 M potassium phosphate buffer, pH 6.0; α2-fucosidase from Corynebacterium sp. (Takara Bio, 40 μU) in the 0.1 M sodium phosphate buffer, pH 8.5, for 60 h. β-Galactosyltransferase (Takara Bio, 0.8 mU) reaction was done as described in Amano et al. 2009.

Mass spectrometry

The oligosaccharides were dissolved in pure water and 0.5 μL of each sample was spotted on a MALDI target plate. 2.5-Dihydroxybenzoic acid (DHBA, 0.5 μL) was used as a matrix, which was prepared by dissolving 10 mg/mL in 40% (v/v) CH3CN-water. The dried mixture was measured by using MALDI-QIT-TOFMS (AXIMA-QIT, Shimadzu Biotech) in the negative-ion mode in MS, MS2, and MS3 modes.

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Conflict of interest statement

None declared.

Abbreviation

PBH, pyrene butanoic acid hydrazide.

References


