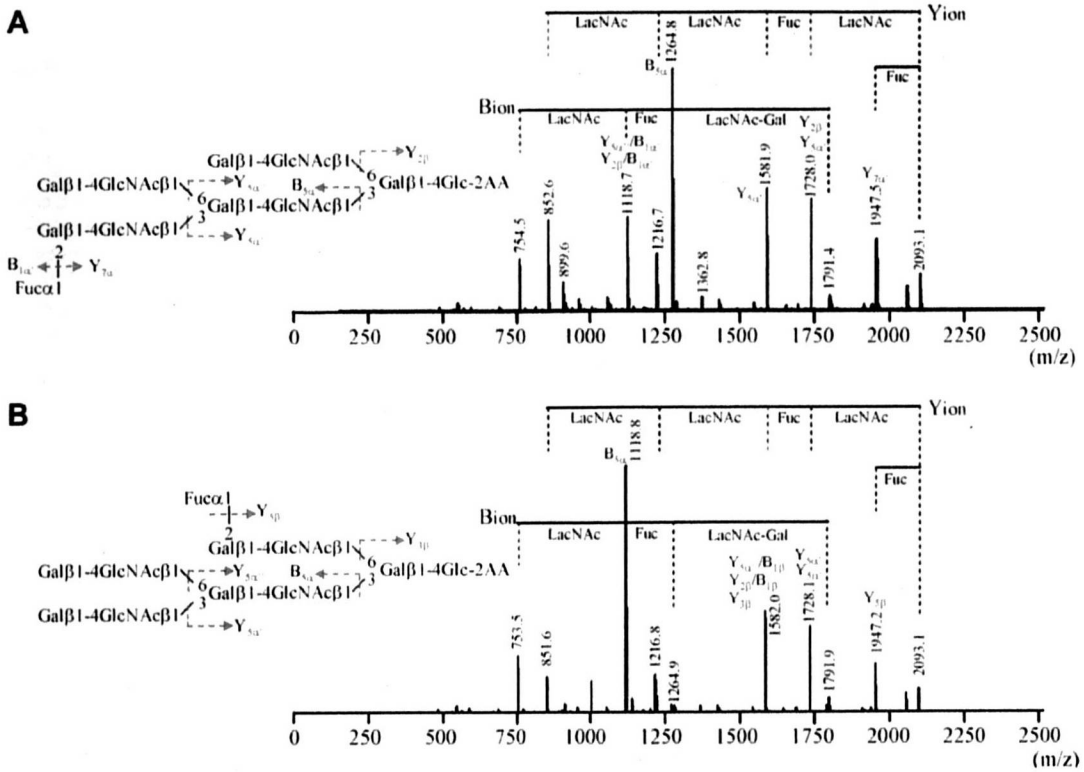


Fig. 5. MS/MS spectra of monofucosyl LNnD HS-7 (A) and HS-8 (B). The dotted lines on the spectra indicate type of cleavage (Y or B ion) according to Domon and Costello's nomenclature [48].

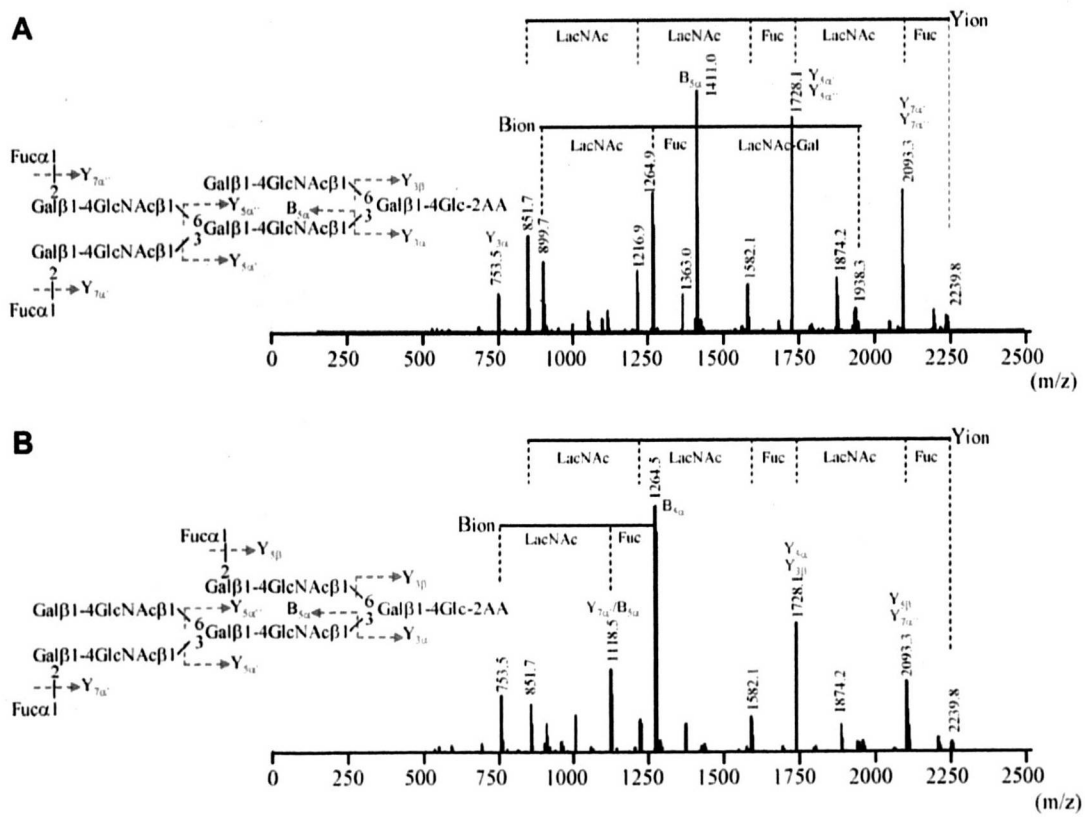


Fig. 6. MS/MS spectra of difucosyl LNnD HS-9 (A) and HS-10 (B). The dotted lines on the spectra indicate type of cleavage (Y or B ion) according to Domon and Costello's nomenclature [48].

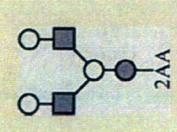
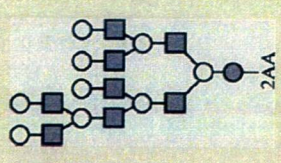
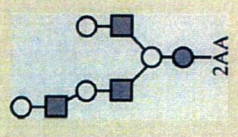
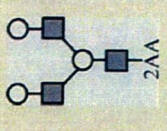
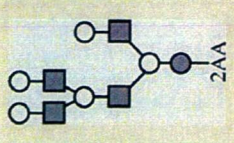
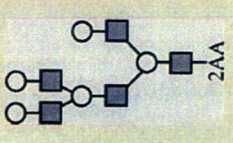
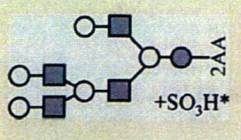
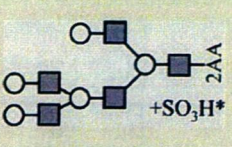
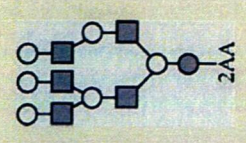
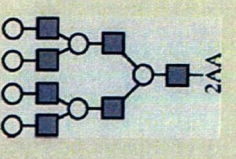
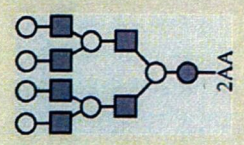
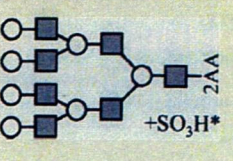
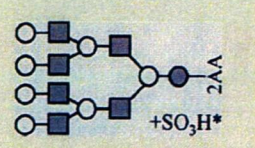
1728.1 and 1582.1 correspond to [H5N3F1–2AA] and [H5N3–2AA], respectively. The Y ion at *m/z* 851.7 corresponds to the composition of $[H3N1–2AA]^+$. These fragment ions of the Y ion series were commonly observed in HS-9 and HS-10. We also observed the set of B ion series, $[H2N2]^+$ at *m/z* 753.5, $[H2N2F1]^+$ at *m/z* 1118.5, $[H3N3F1]^+$ at *m/z* 1264.5, and $[H3N3F2]^+$ at *m/z* 1411.0. A characteristic ion at *m/z* 1411.0 observed in HS-9 (Fig. 6A), which corresponds to three LacNAc units having two Fuc residues, indicates that two Fuc residues are attached to the nonreducing Gal residues of both LacNAc branches linked to Galβ1–4GlcNAcβ1–3Galβ1–4Glc, as shown in Fig. 6A. Thus, the structure of HS-9 is assigned as shown in Fig. 6A. In contrast, a characteristic ion at *m/z* 1264.5 ($B_{5\alpha}$) observed in HS-10 (Fig. 6B) indicates the attachment of only one Fuc to the most outer LacNAc residue. Therefore, the oligosaccharide (HS-10) is assigned to the structure as shown in Fig. 6B.

Among LNnD substituted with α1–2 Fuc residues, HS-7 and HS-10 were abundantly present (Fig. 1B) and both oligosaccharides have an α1–2 Fuc residue on either LacNAc on the α1–3 branch of the LNnH core. These observations suggest that the modification of multibranched core oligosaccharides with α1–2 Fuc residues proceeds preferably at LacNAc residues of the elongated branches.

Discussion

We studied structural features of oligosaccharides from the milk samples of bearded and hooded seals by NP–HPLC and MALDI–TOF MS. The combination of sequential digestion of the oligosaccharides with exoglycosidases and MALDI–TOF MS was a useful technique for elucidation of the branching patterns and modification of oligosaccharides with fucose and/or sulfate group(s). Table 2 shows a list of asialo-oligosaccharides found in bearded and hooded seal milk. The oligosaccharides are categorized into nine core structures (A–I) based on the monosaccharide compositions. LNnD and LNnTD (C and E in Table 2) were observed as common core structures in both milk samples, but the two species showed quite different features. The most characteristic fea-

Table 2
Structural features of the oligosaccharides derived from bearded and hooded seal milk.

ID	Core structure	Number of Fuc residues					ID	Core structure	Number of Fuc residues				
		0	1	2	3	4			0	1	2	3	4
A		HS	HS	–	–	–	F		HS	HS	HS	HS	HS
B		HS	HS	–	–	–	G		–	BS	–	–	–
C		HS	HS BS	HS BS	HS BS	–	H		–	BS	BS	–	–
Cs		–	BS	–	–	–	Hs		–	–	BS	–	–
D		HS	HS	HS	–	–	I		–	–	BS	BS	–
E		HS	HS BS	HS BS	HS BS	HS BS	Is		–	–	–	BS	–
Es		–	–	BS	–	–							

*Core structures with sulfate group at 3-OH position of nonreducing terminal Gal.

ture of oligosaccharides in both milk samples is that multibranched oligosaccharides were present and linear oligosaccharides were not detected in the current study.

BS milk contained characteristic oligosaccharides having monosaccharide compositions of H3N3F1 (G in Table 2), H5N5F1 (H), and H7N7F3 (I), and these oligosaccharides were confirmed as Galβ1–4GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]Galβ1–4GlcNAc and Galβ1–4GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]Galβ1–4GlcNAc. Free oligosaccharides having LacNAc at the reducing end have been reported in bovine and caprine colostrum [33–35]. In mammary glands, lactose is synthesized by lactose synthase, a complex of β4 galactosyltransferase I (βGalT-I) and α-lactalbumin [36]. βGalT-I is also involved in the synthesis of Galβ1–4GlcNAc in the case of the absence of α-lactalbumin. However, α-lactalbumin in lactating mammary glands changes the preferred acceptor of βGalT-I from GlcNAc to Glc [37]. Interestingly, bovine colostrum contains oligosaccharides such as Neu-Acα2-6Galβ1–4GlcNAc, Galβ1–4(Fucα1–3)GlcNAc, and Galβ1–3(Fucα1–4)GlcNAc, but their concentrations decrease dramatically to the trace level 7 days after parturition [38,39]. The presence of large oligosaccharides such as H, HS, I, and Is in BS milk strongly suggests that biosynthesis starts from LacNAc as the core structure.

HS milk contained varieties of oligosaccharides having multibranched core structures (i.e., cores E and F in Table 2). In addition, most oligosaccharides were substituted with different numbers of α1–2 Fuc residues at the nonreducing terminal Gal residues. All oligosaccharides in HS milk have LNnH (Galβ1–4GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]Galβ1–4GlcNAc) as a common core. They are preferentially elongated at the Galβ1–4GlcNAcβ1–3 branch of the LNnH core unit. For example, the core structure having monosaccharide compositions of H6N4 (C in Table 2) has two LacNAc residues on the Galβ1–4GlcNAcβ1–3 branch of LNnH (see Fig. 4B), in contrast to the branching of lacto-*N*-decaose in human milk [40]. Among the β-*N*-acetylglucosaminyltransferase (GnT) family, β3-*N*-acetylglucosaminyltransferase (iGnT), which is a key enzyme for the elongation of LacNAc sequence, prefers type II chain (Galβ1–4Glc/GlcNAc) [41–43]. Urashima and coworkers analyzed small oligosaccharides in HS milk by ^{1}H NMR spectroscopy and revealed that major oligosaccharides contained only type II chains [14]. The addition of Galβ1–4 residue to terminal GlcNAc provides the preferable acceptor for iGnT enzyme. In contrast, HS milk contains multiantennary oligosaccharides (E in Table 2), which have two LacNAc residues on both branches of LNnH, indicating that the Gal residue on the β1–6 branch of LNnH was substituted followed by modification of the β1–3 branch of LNnH. iGnT was considered to be less efficient to the longer LacNAc repeats as the acceptors [44]. Furthermore, the efficiency of iGnT may be decreased by the presence of the Galβ1–4GlcNAcβ1–6 branch to the Gal residue of Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc. In general, the sequence of Galβ1–4GlcNAcβ1–3Galβ1–4Glc/GlcNAc provides the preferred acceptor for β(1–6)*N*-acetylglucosaminyltransferase (IGnT), which is thought to be a key enzyme for the branching of oligosaccharides [45–47]. It is likely that human milk oligosaccharides preferentially is elongated at the Galβ1–4GlcNAcβ1–6 branch of LNH, and the Galβ1–3GlcNAcβ1–3 branch of LNH does not receive further modification with LacNAc [6]. The presence of multibranched oligosaccharides in seal milk suggests that enzyme activities of IGnT and β4GalTs in seal mammary glands are higher than those in other eutherian mammals.

In this article, we have focused on characterization of the branching pattern of oligosaccharides of high molecular masses in seal milk samples by means of the combination of MALDI–TOF MS and sequential exoglycosidase digestion. Branching is one of the major structural features of carbohydrates, and a relatively simple set of monosaccharides can form various branching configurations. Techniques based on MS/MS were used for the structural characterization of oligosaccharides. Special emphasis was made so that the combined use of MALDI–TOF MS and sequential exoglycosidase digestion gave unambiguous structural details of multibranched oligosaccharides, including linkage positions and anomeric configurations.

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