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Structures of the N-linked sugar chains in the PAS-6 glycoprotein from the bovine milk fat globule membrane

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The structures of the N-linked sugar chains in the PAS-6 glycoprotein (PAS-6) from the bovine milk fat globule membrane were determined. The sugar chains were liberated from PAS-6 by hydrazinolysis, and the pyridylaminated sugar chains were separated into a neutral (6N) and two acidic chains (6M and 6D), the acidic sugar chains then being converted to neutral sugar chains (6MN and 6DN). 6N was separated into two neutral fractions (6N13 and 6N5.5), while 6MN and 6DN each gave a single fraction (6MN13 and 6DN13). The structure of 6N5.5, which was the major sugar chain in PAS-6, is proposed to be Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA; 6N13, 6MN13 and 6DN13 are proposed to be Galβ1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 (Galβ1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4 (Fucα1 → 6)GlcNAc-PA; 6M and 6D had 1 or 2 additional NeuAc residues at the non-reducing ends of 6MN13 and 6DN13, respectively.

***Keywords*: PAS-6 glycoprotein, milk fat globule membrane, bovine**

Introduction

The fat globules in milk are enclosed by a membrane which is called the milk fat globule membrane (MFGM). This membrane is derived from the apical plasma membrane of lactating mammary epithelial cells, as has been reviewed [1–4]. MFGM consists of protein, glycoprotein, enzymes, phospholipids, triacylglycerols, cholesterol, glycolipids, and other minor components. MFGM is relatively rich in glycoproteins, of which seven major ones (PAS-1 to -7) have been visualized by the periodate-Schiff reagent (PAS) with SDS-PAGE [2, 5–8]. Of these seven glycoproteins, PAS-6 and PAS-7 have been shown to be very similar by peptide mapping and partial sequencing [9, 10]. There are small distinguishable differences between PAS-6 and PAS-7 in the migration rate with SDS-PAGE (50 kDa and 47 kDa) [9, 11], carbohydrate content (7% and 5.5%) [9], and affinity to Con A [12]. PAS-6 has both N- and O-linked sugar chains, while PAS-7 has only N-linked sugar chains [9]. The differences in these glycoproteins are probably due to differing glycosylation in the mammary gland. However, the structure of the carbohydrate chains of these glycoproteins has still not been elucidated. The carbohydrate moiety of MFGM glycoproteins derived from mammary secretory cells may be concerned with the secretion of milk. Resolution of the structures of the sugar chains of MFGM glycoproteins is necessary to elucidate the structure-function relationship of the sugar chain moiety of the glycoproteins from MFGM. In the present study, we determined the structures of the N-linked sugar chains of the PAS-6 glycoprotein from MFGM.

Materials and methods

Materials

Milk was obtained from Holstein cows of the university herd. Dowex 50Wx2 (200–400 mesh) was from Dowex, AG3-X4A was from Bio-Rad (USA), the TSK gel DEAE-5PW column (0.75 dia. × 7.5 cm, 5 μm) was from Tosoh Corp. (Japan), the GS220 column (0.76 dia. × 50 cm, 9 μm) was from Asahi Kasei (Japan), and the Cosmosil 5C18-P column (0.46 dia. × 15 cm, 5 μm) was from Nakarai (Japan). Phenylmethylsulfonyl fluoride (PMSF), a molecular weight marker protein kit, α-methyl-D-mannopyranoside, *N*-acetylneuraminic acid, *N*-acetylneuramin-lactose, and neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* were all purchased from Sigma (USA). β-Galactosidase (EC 3.2.1.23) from *Aspergillus* sp. was from Toyobo Co. (Japan), while Sephacryl S-200 (superfine) was from Pharmacia Biotech (Sweden). α-Galactosidase (EC 3.2.1.22) from green coffee beans, α-mannosidase (EC 3.2.1.24) from Jack

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bean and β-mannosidase (EC 3.2.1.25) from *Achatina fulia*, α-L-fucosidase (EC 3.2.1.25) from bovine epididymis, β-*N*-acetylhexosaminidase (EC 3.2.1.30) from Jack bean, the PA-glucose oligomer (4–20 units), standard PA-oligosaccharides, and Con A-agarose were all obtained from Seikagaku Kogyo (Japan).

Preparation of MFGM and purification of the PAS-6 glycoprotein

Method 1 of Kanno and Kim [13] was adapted for MFGM preparation. The PAS-6 glycoprotein was purified from MFGM by selective extraction with urea and KCl, gel filtration on Sephacryl S-200, and affinity chromatography on Concanavalin A-agarose, as described in [9]. PAS-6 bound strongly to Con A was recovered by eluting with α-methyl-D-mannopyranoside. SDS-PAGE, using 10% acrylamide [14], resulted in the purified PAS-6 glycoprotein giving a single protein band corresponding to a molecular mass of 50 kDa.

Preparation and pyridylamination of the N-linked sugar chains

The sugar chains of the PAS-6 glycoprotein (10 mg) were liberated by hydrazinolysis (12 h at 100 °C) by using Hydraclub S-204 (Hohnen Corp., Japan) [15]. The excess hydrazine was removed by repeated evaporation with toluene, and the free amino groups were N-acetylated by using sodium bicarbonate and acetic anhydride. The solution was adjusted to pH 3 and added to a small column of Dowex 50W × 2 (H^+ form), and the column washed with five volumes of distilled water. The effluent and washings were combined and dried under reduced pressure over P_2O_5. The reducing ends of the sugar chains were pyridylaminated according to Hase *et al.* [16], the excess reagents being removed by washing at least seven times with two volumes of benzene [17].

Purification of the PA-sugar chains

The PA-sugar chains were fractionated by HPLC in a DEAE-5PW column. The PA-sugar chain fraction that passed through this column was collected and, after being concentrated, was re-analysed in the DEAE-5PW column.

HPLC of the PA-sugar chains

A Spectra-Physics SP8800 HPLC instrument equipped with a Hitachi F-1050 fluorescence spectrophotometer (excitation at 320 nm and emission at 400 nm) and SP 4290 integrator was used. Ion-exchange HPLC was performed in the DEAE-5PW column (0.75 dia. × 7.5 cm) [18]. Solvent A was distilled water adjustd to pH 9 with ammonium hydroxide, and solvent B was a 0.5 M acetic acid-ammonium buffer (pH 8.0). Elution was performed at room temperature with a gradient of solvent B in solvent A, from 0% at 10 min, 25% at 20 min, 25% at 40 min, 35% at 50 min, and 35% at 70 min at a flow rate of 0.5 ml min^{-1}. Reversed-phase HPLC was carried out in a Cosmosil 5C18-P (C18) column (0.46 dia. × 15 cm) [19], elution being performed at room temperature at a flow rate of 1.5 ml min^{-1}, where solvent A was 0.1 M acetic acid, and solvent B was solvent A containing 0.5% *n*-butanol, before the column was equilibrated with a mixture of solvent A and B (70:30, v/v). The proportion of solvent B was linearly increased to 100% over 14 min. Size-fractionation HPLC was carried out in a GS220 column (0.75 dia. × 50 cm, 2 columns joined). Elution was performed at 55 °C at a flow rate of 0.5 ml min^{-1}, using distilled water adjusted to pH 6.0 as the solvent.

Sugar composition analysis

Neutral and amino sugars were analysed by the method of Hase *et al.* [16] as previously described [9]. Sialic acid was determined by the method of Powell and Hart [20], using reversed-phase HPLC in a Capcell Pak C-18 column (0.25 dia. × 25 cm). Elution was carried out with a mixture of distilled water: methanol: sodium perchlorate (2:3:5, v/v/v), and the peak was detected at 549 nm with NeuAc as a standard.

Exoglycosidase digestion

The PA-sugar chains (500 pmol) or their PA-oligosaccharides were digested [21] with α-galactosidase (0.25 U) in 50 μl of a 150 mM citrate-phospate buffer (pH 6.5) in the presence of γ-galactonolactone (1 mg ml^{-1}), while β-galactosidase (0.5 U) digestion was done in 20 μl of a 100 mM acetate buffer (pH 3.6). Treatment with β-*N*-acetylhexosaminidase (0.02 U) was performed in a 100 mM citrate-phosphate buffer (pH 5.0), with α-L-fucosidase (0.02 U) in a 100 mM citrate-phosphate buffer (pH 6.0), and with α-mannosidase (0.2 U) in a 50 mM acetate buffer (pH 4.5). The acidic sugar chain fractions eluted from DEAE-5PW were adjusted to pH 5.0 with 1 M citric acid, concentrated, and then digested with sialidase (0.1 U) in a 0.1 M acetate buffer (pH 5.0). Each digestion was carried out at 37 °C for 16 h in the presence of a few drops of toluene. The reaction was stopped by heating the mixture in a boiling-water bath for 2 min, and then a portion of each digest was analysed by HPLC.

Methylation analysis

The PA-sugar chains (20 nmol) were permethylated by the method of Hakomori [22]. An analysis of partially methylated alditol acetate in chloroform was performed with a Finningan INCOS 50 mass spectrometer connected to a Hewlett-Packard-5992A gas chromatograph equipped with a DB-1 column (0.25 mm dia. × 15 m, 0.25 mm membrane thickness). The temperature was programmed from 150 °C to 200 °C at 2 °C per min. Peaks were identified by referring to those of standard compounds.

Acetolysis

The PA-sugar chains were completely acetylated by adding 40 μl of a mixture of acetic anhydride and pyridine (1:1, v/v) for 15 min at 100 °C, before evaporating to dryness and adding 1 ml of toluene [23]. Partial acetolysis was performed by adding 20 ml of a mixture of acetic acid: acetic anhydride: pyridine (10:10:1, v/v/v) to the completely acetylated PA-sugar chains, the mixture then being allowed to stand for 12 h at 37 °C. The final residue was dissolved in 4 ml of chloroform, and divided into two portions. For an analysis of the reduced terminal of PA-oligosaccharide, one portion was dried, deacetylated by hydrazinolysis (22 h at 100 °C), and then reacetylated (30 min). For an analysis of the non-reduced terminal chain, the other portion was deacetylated with sodium methylate (30 min), dried, and pyridylaminated as already mentioned.

Results

Separation of the PA-sugar chains from the PAS-6 glycoprotein

The PAS-6 glycoprotein purified from MFGM showed a single band with a molecular weight of 50 000 by SDS-PAGE after Coomassie brilliant blue R-250 and periodic acid-Schiff reagent staining (Figure 1). Purified PAS-6 glycoprotein was subjected to hydrazinolysis and followed by pyridylamination.

The purified PA-sugar chains were separated into one neutral sugar chain (6N) and two acidic chains (6M and 6D) in addition to salts shown by the X mark (Figure 2A). The ratio of the peak areas of 6N, 6M and 6D was 67:29:4, respectively. An aliquot of the 6M and 6D peaks was digested with sialidase, which resulted in a shift of the peak to the position of the 6N peak (Figure 2B) and 6DN peak (Figure 2C). It was revealed that 6M and 6D peaks contained monosialyl and disialyl sugar chains, respectively. The acidic sugar chains were converted into neutral sugar chains by neuraminidase digestion and subjected to further analyses. The 6N from 6M and 6N from 6D fractions are expressed as 6MN and 6DN, respectively, hereafter.

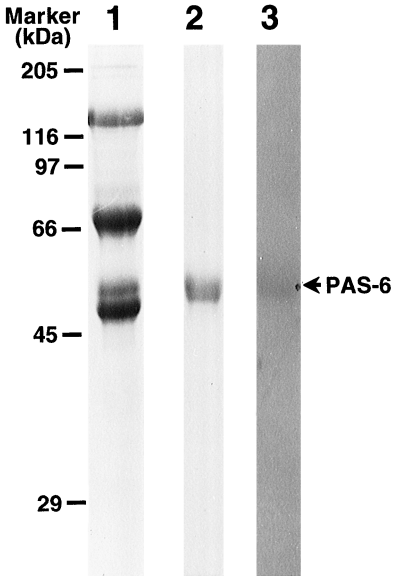


Figure 1. SDS-PAGE patterns of purified PAS-6 glycoprotein. Lane 1, MFGM; lanes 2 and 3, PAS-6 glycoprotein. Lanes 1 and 2 were stained with Coomassie brilliant blue R-250 and lane 3 with periodic acid-Schiff reagent. The loaded protein was 60 μg for MFGM and 20 μg for PAS-6.

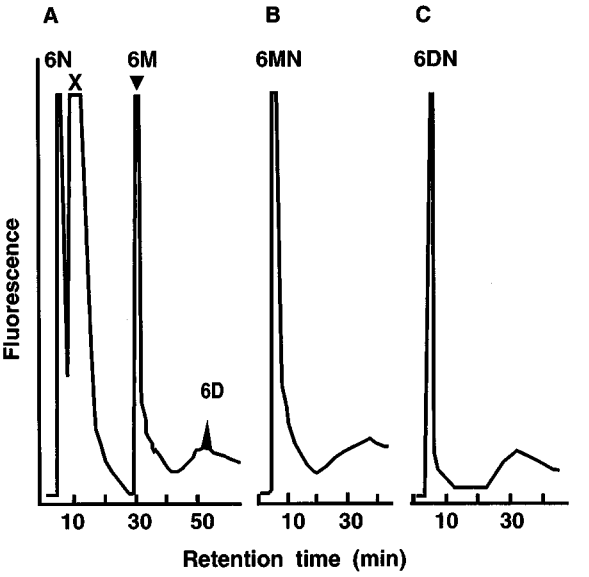


Figure 2. Separation of the PA-sugar chains obtained from the PAS-6 glycoprotein by HPLC in a DEAE-5PW column. A, PA-sugar chains; B, neuraminidase digest of 6M in A; C, neuraminidase digest of 6D in A, N, neutral PA-sugar chains; M, monosialo PA-sugar chains; D, disialo PA-sugar chains. X indicates the position of salts from the reagents. The arrow heads indicate the position of *N*-acetylneuramin-lactose-PA. A shaded area of 6D was integrated.

Size fractionation of the PA-sugar chains from PAS-6

The neutral sugar chains (6N, 6MN and 6DN) were analysed in both a GS220 gel filtration column and reversed-phase C18 column (Figure 3). 6N was separated into two peaks corresponding to GU13.0 and GU5.5 in the GS220 column (Figure 3A), and also into two peaks corresponding to GU13.2 and GU7.1 in the C18 column (Figure 3B). GU13.0 and GU5.5 in the GS220 column corresponded to GU13.2 and GU7.1 in the C18 column, respectively. The ratio of GU13 and GU5.5 was 1:3. 6MN gave a single peak in the GS220 and C18 columns, corresponding to GU13.0 and GU13.2, respectively (Figure 3C and 3D). 6DN also gave a single peak corresponding to GU13 in both columns (data not shown); that is, 6MN and 6DN were eluted at the same position as that of GU13 from 6N by HPLC in both columns. In this paper, we identify the two neutral sugar

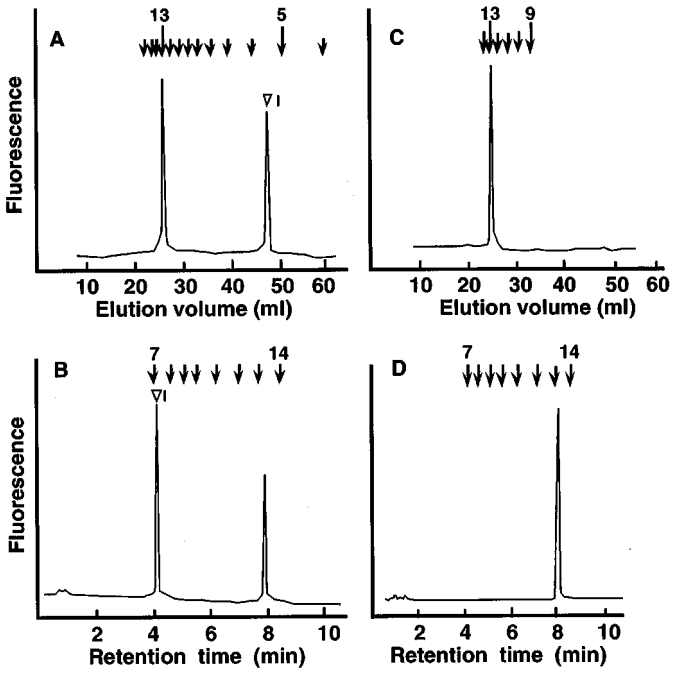


Figure 3. Size-fractionation and separation of the neutral sugar chains of 6N (A and B) and 6MN (C and D) by HPLC in GS220 (A and C) and reversed-phase C18 columns (B and D). The arrows indicate the eluting position of the glucose oligomer and the number of glucose units. The open arrow heads indicate the positions of the standard sugar chain: I, Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA.

Table 1. Sugar compositions of the sugar chains liberated from the PAS-6 glycoprotein

	PA-sugar chains		
	6N13	*6N5.5*	*6M*
Fuc	0.8	0	0.9
Man	3.2	2.9	3.0
Gal	3.8	0	3.9
GlcNAc	2.6	0.8	3.0
PA-GlcNAc	1	1	1
NeuAc	0	0	0.9

Values are presented as the molar ratio to PA-GlcNAc.

chains from 6N as 6N13 and 6N5.5 and, similarly, that from 6MN as 6MN13 and that from 6DM as 6DN13.

Sugar composition of 6N13, 6N5.5 and 6M

The sugar compositions of 6N13, 6N5.5 and 6M are shown in Table 1. The sugar compositions of 6N13 and 6M were very similar, except that 6M contained sialic acid. 6N5.5 did not contain Fuc, Gal and NeuAc. The sugar content of 6D was not determined because of the small amount of the sample.

Exoglycosidase digestion

PA-sugar chains 6N13, 6N5.5, 6MN13 and 6DN13 were digested with exoglycosidases, and each digest was then analysed in both the GS220 and C18 columns.

1) 6N13

Sequential digestion of 6N13 with α-L-fucosidase, β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase produced a new peak at each step (Figure 4 and Table 2). With α-L-fucosidase, 6N13 was produced at the position corresponding to GU11.5, indicating the release of one Fuc residue (Figure 4B). The β-galactosidase digest of GU11.5 was eluted at the position of GU8.5, suggesting that 4 Gal residues had been released (Figure 4C). The β-*N*-acetylhexosaminidase digest of GU8.5 was eluted at the position of GU5.5, indicating that 2 GlcNAc residues had been released, its position being the same as that of Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA (Figure 4D). Finally, α-mannosidase digestion of GU5.5 produced

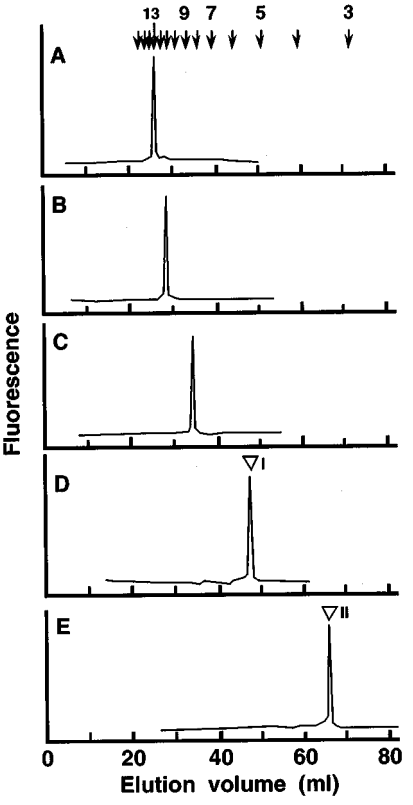


Figure 4. Sequential exoglycosidase digestion of the neutral sugar chain of 6N13 by size-fractionation HPLC in a GS220 column. A, 6N13; B, α-fucosidase digest of A; C, β-galectosidase digest of B, D, β-*N*-acetylhexosaminidase digest of C; E, α-mannosidase digest of D. The arrows indicate the eluting position of the glucose oligomer and the number of glucose units. The open arrow heads indicate the positions of the standard sugar chains: I, Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA; II Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA.

Table 2. Summary of the sequential digestion with exoglycosidase of sugar chains from the PAS-6 glycoprotein

Sugar chain	*Enzyme*[a]	*Substrate*	*Elution*[b] *position (GU)*	*Number of released sugar residues*
6N13	1) *α*-L-Fucosidase	6N13	11.5	1 Fuc
6MN13	2) *β*-Galactosidase	GU11.5	8.5	4 Gal
6DN13	3) *β-N*-Acetylhexosaminidase	GU8.5	5.5[c]	2 GlcNAc
	4) *α*-Mannosidase	GU5.5	3.5[c]	2 Man
6N5.5	*α*-Mannosidase	6N5.5	3.5[c]	2 Man

[a]These enzymes were sequentially used.
[b]This shows the number of glucose units (GU) corresponding to the elution position from the GS220 gel filtration column.
[c]GU5.5 was eluted at the same position as that of Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA, and GU3.5 at the same position as that of Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA.

a new peak at the position of GU3.5 and at the same position as that of Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA (Figure 4E). The PA-oligosaccharides of GU13 and GU11.5 before and after α-fucosidase digestion could not be digested with α-galactosidase from coffee bean (data not shown). The sugar chain of 6N13 was composed of αFuc (1) and βGal (4) at the non-reducing ends, and of αMan (2), βMan (1), βGlcNAc (3) and GlcNAc-PA (1) residues, the sequence being determined as Fucα1 → [(Galβ1 →)$_4$(GlcNAcβ1 →)$_2$ (Manα1 →)$_2$Manβ1 → GlcNAcβ1 →]GlcNAc-1

2) 6N5.5

6N5.5 could not be sequentially digested with α-L-fucosidase, β-galactosidase, and β-*N*-acetylhexosaminidase. The α-mannosidase digest of 6N5.5 was eluted at the position of GU3.5 and Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA, indicating the release of 2 Man residues (Table 2). The ratio of Man to GlcNAc (3:2) was similar to that shown for the composition of 6N5.5 in Table 1. These results suggest that 6N5.5 was composed of αMan 2, βMan 1, βGlcNAc 1 and GlcNAc-PA 1 residues, the sequence being determined as 2Manα → Manβ → GlcNAcβ → GlcNAc-PA.

3) 6MN13

Sequential digestion of 6MN13 with α-L-fucosidase, β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase resulted in one Fuc, four Gal, two GlcNAc and two Man residues being released by the respective enzymes (Table 2). The digestion products eluted at the GU5.5 and GU3.5 positions were at the same positions as that of Manα1 → 6 (Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA and Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA, respectively. Coffee bean α-galactosidase did not digest the PA-sugar chain of GU13 before α-fucosidase digestion (data not shown). The results for 6MN13 were identical to those for 6N13.

4) 6DN13

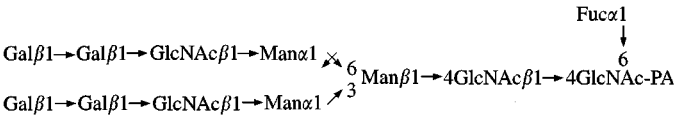
6DN13, the disialo acidic sugar chain, was similarly digested (Table 2), the sequence of 6DN13 being identical to that of 6N13 and 6MN13.

Partial acetolysis of the sugar chains

The foregoing results suggested that the neutral sugar chains of 6N13, 6N5.5, 6MN13, and 6DN13 had the core structure of an N-linked sugar chain. The PA-sugar chains were thus re-pyridylaminated after partial acetolysis and analysed by HPLC.

1) 6N13

The reducing terminal sugar chain obtained by the partial acetolysis of 6N13 was eluted at the position of GU9.1 (Figure 5A-1), while the β-galactosidase digest of GU9.1 was eluted at the GU7.5 position, suggesting the release of two βGal residues (Figure 5A-2). The sugar sequence of GU9.1, therefore, is considered to be Galβ → Galβ → GlcNAcβ → Manα1 → 3Manβ → GlcNAc → GlcNAc-PA. Similarly, the non-reducing terminal PA-sugar chain was eluted at the position of GU3.8, and the β-galactosidase digest was shifted to the GU2.3 position, suggesting that 2 βGal residues were also released (Figure 5B). Partial acetolysis cleaved the 1 → 6 linkage more selectively than the 1 → 3 linkage at a branching point. The non-reducing terminal of the GU3.8 sugar chain is thus likely to possess the sequence of Galβ → Galβ → GlcNAcβ → Manα1 → 6. These results suggest that there were 2 βGal residues at the non-reducing terminal in the biantennary chain of 6N13. The sequence and branching linkage is suggested to be that shown below, in combination with the results of sequential exoglycosidase digestion.



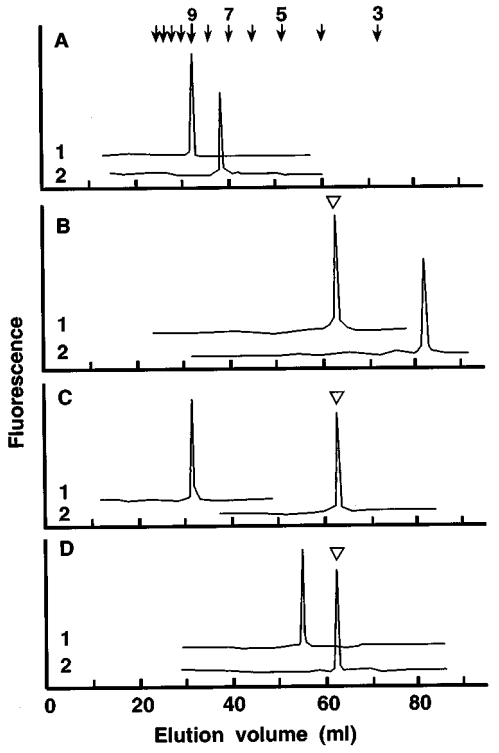


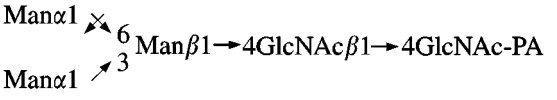
Figure 5. HPLC patterns for size-fractionation in a GS220 column of the PA-sugar chains after partial acetolysis of 6N13, 6MN13 and 6N5.5. A-1, reducing end of 6N13; A-2, β-galactosidase digest of 6N13 from A-1; B-1, non-reducing end of 6N13; B-2, β-galactosidase digest of 6N13 from B-1; C-1, reducing end of 6MN13; C-2, non-reducing end of 6MN13; D-1, reducing end of 6N5.5; D-2, α-mannosidase digest of 6N5.5 from D-1. The arrows indicate the eluting positions of the glucose oligomers and the number of glucose units. The open arrow heads indicate the positions of standard sugar chain Manβ1 → 4GlcNAcβI → 4GlcNAc-PA.

2) 6MN 13 and 6DN13

The reducing terminal product obtained by the partial acetolysis of 6MN13 or 6DN13 gave the peak at the GU9.1 position, while the non-reducing terminal product was at the GU3.8 position (Figure 5C). 6MN13 and 6DN13 were thus constructed with a biantennary chain having the same sugar sequence as that of 6N13.

3) 6N5.5

The reducing terminal chain of 6N5.5 was eluted at the position of GU4.6, and the α-mannosidase digest corresponded to GU3.8 and Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA (Figure 5D). The non-reducing terminal chain was eluted at the position of GU0.8, indicating this to be a monosaccharide, which was further confirmed to be Man by a sugar analysis with HPLC. The sequence of 6N5.5 was determined to be that shown below.



Methylation analysis

Permethylated alditol acetates derived from the PA-sugar chains of 6N13, 6MN13 and 6N5.5 were analysed by a gas chromatography-mass spectrometry, the results being summarized in Table 3. Methylation of 6N13, 6MN13 and 6N5.5 indicated the presence of a single branch involving a Man residue substituted at positions 3 and 6 by 2,4-di-*O*-methylmannitol. Regarding the four Gal residues of 6N13

Table 3. Methylation analysis of the PA-sugar chains released from the PAS-6 glycoprotein from MFGM

	6N5.5	*6N13 and 6MN13 (Mole)*
Fucitol		
2,3,4-Tri-*O*-methyl-1,5-di-*O*-acetyl-	ND	0.92 (1)
Mannitol		
2,4-Di-*O*-methyl-1,3,5,6-tetra-*O*-acetyl-	2.72 (1)	1.32 (1)
2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl-	5.40 (2)	ND
3,4,6-Tri-*O*-methyl-1,2,5-tri-*O*-acetyl-	ND	2.54 (2)
Galactitol		
2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl-	ND	2.40 (2)
2,4,6-Tri-*O*-methyl-1,3,5-tri-*O*-acetyl-	ND	2.38 (2)
Acetamido-2-deoxyglucitol		
3,6-Di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methyl-	2.60 (1)	3.92 (3)
3,6-Di-*O*-methyl-4,5-di-*O*-acetyl-1-deoxy-1-(2-pyridyl-methylamino)-2-*N*-methyl-	2.26 (1)	ND
3-Mono-*O*-methyl-4,5,6-tri-*O*-acetyl-1-deoxy-1-(2-pyridyl-methylamino)-2-*N*-methyl-	ND	1.01 (1)

Values in the parentheses are expressed by taking the value of 2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylmannitol as 1.0.

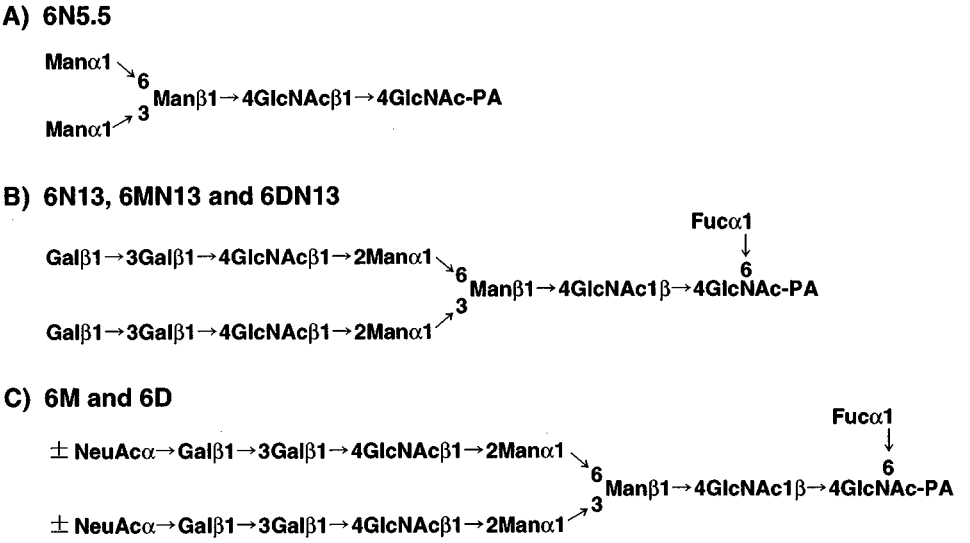


Figure 6. Structures of the sugar chains of the PAS-6 glycoprotein. In 6M, one NeuAc residue is added to either outer Gal, and in 6D, two NeuAc residues are in both outer Gal.

and 6MN13, two residues were at the non-reducing terminal and were found to be linked at position 1 by 2,3,4,6-tetra-*O*-methylgalactitol, while the remaining two residues were linked at positions 1 and 3 by 2,4,6-tri-*O*-methyl galactitol. The two Man residues of 6N5.5 were at the non-reducing termini and were found to be linked at position 1 by 2,3,4,6-tetra-*O*-methylmannitol. A fucose residue was also at the non-reducing terminus and linked to the 6 position of the reducing-terminal GlcNAc residue.

Structure of the sugar chains of 6N5.5, 6N13, 6MN13 and 6DN13

From the foregoing results, the structures of the neutral sugar chains of 6N5.5, 6N13, 6MN13, and 6DN13 are proposed as shown in Figure 6. In addition, the acidic sugar chains of 6M and 6D contained one and two NeuAc residues in addition to those of 6MN13 and 6DN13, respectively (Figure 6). The molar ratio of 6N13:6N5.5:6M:6D was 17:50:29:4, the 6N5.5 sugar chain thus being the major one in the PAS-6 glycoprotein.

Discussion

The PAS-6 glycoprotein of bovine MFGM was shown in this study to contain four N-linked sugar chains. To analyse the size of the PA-sugar chains, we used an Asahi Pak GS220 gel filtration column for HPLC, instead of the amide column or Bio Gel P-4 column that are widely used. By joining two GS220 columns (100 cm), glucose oligomers of up to 20 U could be separated within 140 min (data not shown). The elution positions of the standard PA-sugar chains are shown in Table 4 in comparison with those in an Amide-80 column [24]. With the GS220 column, the elution positions of Man, Gal, GlcNAc and Fuc corresponded to 0.8, 0.75, 1.5, and 1.5 glucose units, while those with the Amide-80 column were 1.0, 1.0, 0.5 and about 0.5 glucose units, respectively. The elution time for the sugar chains in the GS220 column was longer (about two times) than that in the Amide-80 column, but shorter than that in the Bio Gel P-4 column. A characteristic of the GS220 column is that distilled water can be used as the solvent, which is easier to evaporate for concentration and does not need deionizing as is required with the Amide-80 column. The GS220 column could be successfully used for size fractionation before and after exoglycosidase digestion.

All of the N-linked sugar chains had a biantennary chain with the core structure that is a common characteristic of N-linked sugar chains. The neutral chain of 6N5.5 had the simple structure of 2Manα (1 → 6,1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA and constituted 47% of all the sugar chains of the PAS-6 glycoprotein. This chain is the core structure of N-linked sugar chains, but has not been found in any intermediates on the major biosynthetic pathway after processing from Glc3-Man9-GlcNAc2 [25]. The Manα1 → 6(Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc group seems to be that converted after processing Glc3-Man3 from Glc3-Man5-GlcNAc2, which has been synthesized on the alternate pathway for a complex-type oligosaccharide [25] by the action of α-glucosidase I and II and α-mannosidase I specific for Manα1 → 2Man. This alternate biosynthetic pathway for N-linked oligosaccharides has been found in hamster ovary cells [26] and BHK cells [27] with glucose starvation, in Thy-1$^-$ mutant mouse lymphoma cells [28, 29], and ovomucoid from hen [30] and Japanese quail [31]. This resulted from an inability

Table 4. Comparison of the elution positions of the PA-sugar chains from the GS220 and Amido 80 columns

PA-sugar chain	*Amido 80*[a] *(Glucose unit)*	*GS220*
Mα1 → 6(Mα1 → 3)M1β → R1	4.3	5.4
GNβ1 → 4Mα1 → 6(GNβ1 → 4Mα1 → 3)M1β → R1	5.1	8.4
Gβ1 → 4GNβ1 → 4Mα1 → 6(Gβ1 → 4GNβ1 → 4Mα1 → 3)M1β → R1	7.0	9.9
Gβ1 → 4GNβ1 → 4Mα1 → 6(Gβ1 → 4GNβ1 → 4Mα1 → 3)M1β → R2	7.4	11.4
Gβ1 → 4GNβ1 ↘ 4 Mα1 2 ↘ 6 Gβ1 → 4GNβ1 ↗ 3 M1β → R2 Gβ1 → 4GNβ1 → 4Mα1 ↗	8.6	13.7

M, Man; GN, GlcNAc; G, Gal; R1, GlcNAcβ1 → GlcNAc-PA; R2, GlcNAcβ1 → (Fucα1 → 3)GlcNAc-PA.
[a]Cited from Takahashi [24].

to synthesize dolichol-P-Man [32]. It has been reported that the Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc group constituted only a few per cent of the sugar chain found in endoglycosidase H-resistant oligosaccharides from human β-glucuronidase [33] and in ovomucoid from hen [30] and Japanese quail [31]. It was suggested that this kind of oligosaccharide might have originated from incompletely processed or degraded complex oligosaccharides. The present results indicate, however, that the N-linked sugar chains of PAS-6 were predominantly synthesized via the alternate biosynthetic pathway, rather than the degradation products via the major pathway in bovine mammary epithelial cells. *N*-acetylglucosaminyltransferase I from bovine colostrum is active towards the Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAβ1 → 4GlcNAc group [34]. The biantennary complex-type sugar chains of 6N13 may, therefore, be synthesized from the Manα1 → 6 (Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4GlcNAc group. The addition of β-GlcNAc residues to the trimannosyl core occurs rather randomly and the formation of the complex type sugar chains starting from Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc-Asn is not so strictly regulated as those from Manα1 → 6 (GlcNAcβ1 → 2Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc-Asn [31].

The sequence and anomeric configuration of Galβ1 → 3Galβ1 → 4GlcNAcβ1 was confirmed by the parallel use of α- and β-galactosidase digestion of 6N13, 6MN13 and 6DN13 before and after partial acetolysis and by a methylation analysis. The β-galactosidase used in this study was free from α-galactosidase. The anomeric configuration of the Galα1 → 3Galβ1 → 4GlcNAc group in the N-linked sugar chains has been found in calf thymocyte plasma membrane glycoprotein [35], subcomponent C1q of bovine complement [35], tyroglobulin from calf [36] and from several mammalian sources except human [37], and in the glycoprotein of the Friend murine leukaemia virus [38]. Interestingly, Galα1 → 3Galβ1 → 4GlcNAc was not found to be an acceptor for α2 → 6 sialytransferase from bovine colostrum, since α1 → 3 galactosyltransferase competes with α2 → 6 sialytransferase [39]. It is considered, therefore, that the terminal Gal residue in the Gal → Gaβ1 → 4GlcNAc group of the sugar chains of 6M and 6D, which contained one or two NeuAc residues, was not α- but β-galactosylated.

In addition, the GalNAcβ1 → 4GlcNAc group instead of the Galβ1 → 4GlcNAc group in their outer chain moieties have been reported in a portion of the bi-, tri- and tetra-antennary hybrid- and complex-type sugar chains of PAS-4 and -5 glycoproteins from MFGM [40, 41]. The N-linked sugar chains found in PAS-6 were not in the sugar chains of PAS-4 and -5, and the sugar chains of PAS-4 and -5 were not in PAS-6. Galactosyltransferase or *N*-acetylgalactosaminyltransferase acts specifically in the bovine mammary epithelial cell after *N*-acetylglucosaminylation in the N-linked biantennary sugar chains by recognizing either the complex or hybrid type of oligosaccharides and protein as the substrate. Structures of N-linked sugar chains in the PAS-7 glycoprotein are analysed.

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