Structures of the N-Linked Sugar Chains in PAS-7 Glycoprotein Sharing the Same Protein Core with PAS-6 Glycoprotein from the Bovine Milk Fat Globule Membrane

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Glycoproteins PAS-6 (50 kDa) and -7 (47 kDa) from the bovine milk fat globule membrane share a common protein core but differ in their carbohydrate moiety. We here analyzed and proposed the structures of the N-linked sugar chains of PAS-7. The N-linked sugar chains were liberated from PAS-7 by hydrazinolysis and, after modifying the reducing ends with 2-aminopyridine (PA), were separated into one neutral (7N, 55%) and two acidic (7M, mono-, 43%; 7D, di-, 2%) sugar chain groups. The latter were converted into neutral groups (7MN and 7DN) by sialidase digestion. 7N was finally separated into 5 chains (7N1A, 7N1B-1, 7N1B-2, 7N2A, and 7N2B), and 7MN and 7DN were separated into 3 (7MN1, 7MN2, and 7MN3) and 2 (7DN1 and 7DN2) chains, respectively. The structure of each of these PA-neutral sugar chains was determined by sugar analysis, sequential exoglycosidase digestion, partial acetylation, and 1H-NMR spectroscopy. The results show that the 10 sugar chains were of the biantennary complex type with and without fucose. The structure of 7N2A, one of the major sugar chains, was proposed as:

\[ \text{Galβ1-4GlcNAcβ1-2Manα1\textsuperscript{6}} \]  
\[ \text{Manβ1-4GlcNAcβ1-4GlcNAc-PA} \]

A structural comparison between PAS-6 and -7 indicated that, although they shared the same protein core, their sugar moiety was markedly different, involving the existence of a different pathway during the post-transcriptional modification.

Key words: PAS-7 glycoprotein; milk fat globule membrane; bovine; N-linked sugar chain

During milk secretion, projection of the surface membrane into the alveolar lumen by enveloping intracellular lipid droplets with the apical plasma membrane is one of the most remarkable aspects of biological membrane action throughout nature. Lipid droplets synthesized in the mammary epithelial cells are extruded by being enveloped with a portion of the apical plasma membrane. The membrane surrounding the lipid droplet is referred to as the milk fat globule membrane (MFGM). This membrane contains proteins to about 50%, which have an important function in maintaining the integrity of the membrane, keeping the emulsion stable, and protecting the lipid from attack by lipoprotein lipase in the milk. In addition, some of the MFGM glycoproteins seem to act as a multifunctional adhesion protein in the mammary epithelial cell. MFGM is relatively rich in glycoproteins, of which seven major ones (PAS-1 to -7) have been identified by SDS-PAGE and are made visible by periodic acid-Schiff reagent (PAS) staining. Of these seven glycoproteins, PAS-6 and PAS-7, corresponding to components 15 and 16 of Mather and Keenan, have been demonstrated to be glycosylation variants sharing a common protein core, the cDNA of which has been cloned. PAS-6/7 has two EGF domains in the N-terminal region, the second of which contains an RGD cell adhesion sequence motif. There are distinguishable differences between PAS-6 and PAS-7 in their migration rate by SDS-PAGE (50 kDa and 47 kDa), carbohydrate content, and affinity to Con A. These differences are probably due to the differences in carbohydrate moiety. It has been demonstrated that PAS-6 had two kinds of biantennary complex-type sugar chains with a novel α1→3 linked Gal residue at the non-reducing ends of the simple core structure of the N-linked sugar chain. However, the structure of the N-linked sugar chains

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Abbreviations: MFGM, milk fat globule membrane; EGF, epidermal growth factor; HPLC, high-pressure liquid chromatography; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PAS, periodic acid-Schiff reagent; Man, mannose; Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; SA, sialic acid; NeuAc, N-acetylneuraminic acid; PA, 2-aminopyridine; GU, glucose unit
of PAS-7 has not previously been identified. The carbohydrate moiety of MFGM glycoproteins derived from mammary secretory cells may be concerned with the secretion of milk lipid. It would be interesting to know if PAS-6 and -7 bear distinct sugar chains characteristic of each, although consisting of the same polypeptide. Structural resolution of the sugar chains of glycoproteins in MFGM is necessary to discover their structure-function relationship.

In this study, we identified the structures of the N-linked sugar chains in PAS-7 glycoprotein from MFGM and describe the differences in sugar chain structure in comparison with those proposed for PAS-6.

Materials and Methods

**Materials.** Milk was obtained from Holstein cows of our university herd, Dowex 50W × 2 (H+ form, 200–400 mesh) was from Dowex, AG-3X4 (OH− form, 100–200 mesh) was from Bio-Rad (U.S.A.), the Toyopearl HW-40F column (1.6 × 50 cm), TSK gel DEAE-5PW column (0.75 × 7.5 cm) and TSK gel Amide-80 column (0.45 × 25 cm) were from Tosoh Corp. (Japan), the GS220 column (0.75 × 50 cm) was from Asahi Kasei (Japan), and the Cosmosil 5C18-P column (0.45 × 15 cm) was from Nakarai Tesque (Japan). The molecular weight marker protein kit, α-methyl-D-mannopyranoside, N-acetylneuraminidase from *Clostridium perfringens*, N-acetylneuraminidase from Newcastle disease virus, and β-galactosidase from *Aspergillus niger* were all purchased from Sigma (U.S.A.). Sephacryl S-200HR (superfine) was from Pharmacia Biotech (Sweden), and α-L-fucosidase from bovine kidney was from Boeringer Mannheim (Germany). α-Mannosidase and β-N-acetylhexosaminidase from jack bean, the PA-glucose oligomer (4–20 glucose units), and Con A-agarose were obtained from Seikagaku Kogyo (Japan). The following standard PA-oligosaccharides were obtained from Takara Shuzo (Japan): I, Man₁₋₆(Man₃₋₆)Man₁₋₄GlcNAcβ₁₋₄GlcNAc-PA; II, GlcNAcβ₁₋₂Man₁₋₆(6GlcNAcβ₁₋₂Man₁₋₃)Man₁₋₄GlcNAcβ₁₋₄GlcNAc-PA; III, Galβ₁₋₄GlcNAcβ₁₋₂Man₁₋₆(6Galβ₁₋₄GlcNAcβ₁₋₂Man₁₋₃)Man₁₋₄GlcNAcβ₁₋₄GlcNAc-PA; IV, Galβ₁₋₄GlcNAcβ₁₋₂Man₁₋₆(6GlcNAcβ₁₋₂Man₁₋₃)Man₁₋₄GlcNAcβ₁₋₄GlcNAc-PA; monosialo-sugar chain, NeuAco₂₋₆Galβ₁₋₄GlcNAcβ₁₋₂Man₁₋₆(Galβ₁₋₄GlcNAcβ₁₋₂Man₁₋₃)Man₁₋₄GlcNAcβ₁₋₄GlcNAc-PA; and disialo-sugar chain, NeuAco₂₋₆Galβ₁₋₄GlcNAcβ₁₋₂Man₁₋₆(6NeuAco₂₋₆Galβ₁₋₄GlcNAcβ₁₋₂Man₁₋₃)Man₁₋₄GlcNAcβ₁₋₄GlcNAc-PA.

**Purification of PAS-7 glycoprotein from bovine MFGM.** MFGM was prepared by method 1 of Kanno and Kim.[⁷] PAS-7 was purified from MFGM by selective extraction with urea and KCl, gel filtration in a Sephacryl S-200HR column, and affinity chromatography in a Concanavalin A-agarose column.[⁵] PAS-7 bound weakly to Con A was eluted with 50 mM α-methyl-D-mannopyranoside, but PAS-6 was eluted with 500 mM α-methyl-D-mannopyranoside, its purity being ascertained by SDS-PAGE with a 10% acrylamide gel.[⁸]

**Preparation and pyridylation of the N-linked sugar chains of PAS-7.** The N-linked sugar chains of PAS-7 were liberated by hydrazinolysis (6 h at 100°C) and subsequent N-acetylation[⁹] on Prep 1000 (Oxford Glyco System, U.K.). The released oligosaccharides were modified with 2-aminopyridine and reduced with sodium cyanoborohydride.[²⁰] The excess reagents were removed by using a Toyopearl HW40-F column (1.6 × 50 cm) that had been equilibrated with 0.01 M ammonium acetate at pH 6.0, and the pass-through fraction containing the pyridylaminated oligosaccharides was collected and lyophilized.

**Separation of the N-linked sugar chains into neutral and acidic fractions.** The N-linked sugar chains were separated into neutral and acidic fractions by DEAE-5PW HPLC. Elution was done at a flow rate of 0.5 ml/min at room temperature with two solvents (A and B). Solvent A was distilled water adjusted to pH 9.0 with ammonium hydroxide, and solvent B was an 0.5 M ammonium-acetate buffer at pH 8.0. The column was equilibrated with solvent A. After a sample was injected, the proportion of solvent B was increased by a linear gradient to 30% in 10 min and then maintained at 30% for 30 min.[²¹] The fractions were individually collected and lyophilized.

**HPLC of the PA-oligosaccharides.** An SP 8800 pump (Spectra-Physics, U.S.A.), an F-1050 fluorescence spectrophotometer (Hitachi, Japan; excitation at 320 nm and emission at 400 nm) and an SP 4290 integrator (Spectra-Physics, U.S.A.) were used for separating the PA-oligosaccharides. Size-fractionation HPLC was done in a GS220 column (0.75 × 50 cm, 2 columns in series), elution being done at a flow rate of 0.5 ml/min at 55°C using distilled water adjusted to pH 6.0 with ammonium hydroxide as the solvent.[¹⁶] Reversed-phase HPLC with a Cosmosil 5C18-P column (0.46 × 15 cm) was done by eluting at a flow rate of 1.0 ml/min at room temperature with two solvents (A and B). Solvent A was 0.1 M acetic acid and solvent B was 0.1 M acetic acid containing 0.5% 1-butanol. The column was equilibrated with a mixture of solvents A and B of 95:5 by volume. After injecting a sample (50 μl, >50 pmol), the ratio of solvent B to A was increased by a linear gradient to 100% of solvent B in 55 min.[²²] Each oligosaccharide...
fraction (about 1.5 ml) was collected and evaporated to dryness in vacuo. The residue was dissolved in a small amount (about 10-50 µl) of solvent C (described below) and about 10 pmol of the sample was injected into the second column. Size-fractionation HPLC with a TSK gel Amide-80 column (0.46 × 25 cm) was performed at a flow rate of 1.0 ml/min and 40°C with two solvents (C and D). Solvent C was composed of 3% acetic acid in distilled water adjusted to pH 7.3 with triethylamine, and acetonitrile at 35:65 by volume.23 Solvent D was composed of 3% acetic acid in water adjusted to pH 7.3 with triethylamine, and acetonitrile (50:50, v/v). The column was equilibrated with solvent C. After a sample was injected, the ratio of solvent D to C was increased by a linear gradient to 100% of solvent D in 50 min.

Carbohydrate analysis. PAS-6 and PAS-7 were hydrolyzed with 4 M trifluoroacetic acid for 3 h at 100°C under oxygen-free conditions. A carbohydrate analysis was done by combined high-pressure anion-exchange chromatography and pulsed-amperometric detection (HPAEC/PAD, Dionex, U.S.A.).24 The saccharides were eluted isocratically with 16 mM NaOH from a polymeric anion-exchange CarboPac PA1 column (0.4 × 25 cm, Dionex) and detected by a pulsed-electrochemical detector (Dionex, U.S.A.). Alternatively, the neutral and amino sugars were analyzed by the method of Suzuki et al.25 After hydrolysis of the above-mentioned PA-oligosaccharides, the hydrolyzed monosaccharides were again pyridylaminated and re-N-acetylated. The resulting PA-monosaccharides were measured by ion-exchange HPLC in a TSK gel Sugar AXI column (0.46 × 15 cm, Tosoh, Japan).26 Sialic acid was measured and identified by the method of Hara et al.27 using reversed-phase HPLC with a Capcell Pac C-18 column (0.25 × 25 cm, Shiseido, Japan). Elution was done with a mixture of methanol: acetonitrile: distilled water (3:0.5:10, v/v/v), and fluorescence was detected at excitation and emission wavelengths of 373 and 448 nm, respectively. NeuAc was used as a standard.

Sequential exoglycosidase digestion of the PA-sugar chains. The neutral PA-sugar chains (about 100 pmol) were sequentially digested with a series of exoglycosidases in 50 µl of an appropriate buffer at 37°C for 18 h in the presence of a drop of toluene. Digestion with β-galactosidase (0.1 unit) from Aspergillus niger was done in a 0.1 M citrate-phosphate buffer at pH 4.0; with β-N-acetylhexosaminidase (0.1 unit) from jack bean in a 0.1 M citrate-phosphate buffer at pH 5.0; with α-mannosidase (1 unit) from jack bean in a 0.1 M acetate buffer at pH 4.5; and with α-fucosidase (0.03 unit) from bovine kidney in a 0.1 M citrate-phosphate buffer at pH 4.5. The acidic sugar chains that were recovered from DEAE-5PW were adjusted to pH 5.0 with 1 M citric acid, evaporated by a speed vacuum concentrator, and then digested with either sialidase (0.1 unit) from Clostridium perfringens or 0.1 M acetate buffer at pH 5.0, or with sialidase (0.8 unit) from Newcastle disease virus in a 0.05 M sodium cacodylate buffer at pH 6.5. The reaction was stopped by heating for 3 min in a boiling water bath, and a sample of the digest was analyzed by HPLC in an Amide-80 column.23

Partial acetylation. 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 being evaporated to dryness and mixed with 1 ml of toluene. Partial acetylation was done by adding 20 ml of a mixture of acetic anhydride:pyridine (10:1, v/v) to the completely acetylated PA-sugar chains,27 the mixture then being left 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 acetylated (30 min). For an analysis of the non-reduced terminal chain, the other portion was deacetylated with sodium methoxide-methanol solution (30 min), dried, and pyridylaminated as already mentioned. 2-PA non-reduced terminal sugar was measured by anion-exchange column HPLC.25

600-MHz 1H-NMR spectroscopy. The PA-sugar chains were dissolved in 99.8% 2H2O and freeze-dried. This procedure was done three times, and the final freeze-dried materials were dissolved in 0.4 ml of 99.995% 2H2O to make a 5-10 nmol sugar-chain solution. 1H-NMR spectra were recorded by a JMN-ALPHA600 spectrometer (JEOL, Japan) operated at 600-MHz in the Fourier transform mode. Chemical shifts (δ) are with reference to internal standards; acetone set to δ = 2.216 ppm (in 2H2O at 23°C) and 4,4'-dimethyl-4-silapentane-1-sulfonate set to δ = 0. Scanning was done 10,000 times. The NMR spectra were interpreted by comparing with NMR data in the literature.21,28-30

Results

Carbohydrate composition of PAS-7 glycoprotein

The purity of PAS-7 was confirmed by SDS-PAGE. The PAS-6 and PAS-7 preparations gave single protein bands of 50 and 47 kDa, respectively (Fig. 1). Purified PAS-6 and PAS-7 were hydrolyzed, and their carbohydrate composition was analyzed by HPAEC/PAD, the composition of PAS-7 being shown in Table I in comparison with that of PAS-6. The carbohydrate content of PAS-7 was 10.6%, about half that of PAS-6 (16.5%). The sugars of both glycoproteins were identified as N-acetylgalac-
tosamine, N-acetylglycosamine, mannose, fucose, galactose, and N-acetylneuraminic acid. The molar ratio of monosaccharides in PAS-7 was similar to PAS-6, but mannose was identified by a high content in PAS-6, suggesting that the presence of different structures of the sugar chains.

Separation of the PA-sugar chains released from the PAS-7 glycoprotein

The PA-sugar chains were prepared from 5 mg of PAS-7 by hydrazinolysis, N-acetylation and pyridylation, before being separated in a DEAE-5PW column in accordance with the number of sialic acid residues into three fractions; one neutral sugar chain (7N) and two acidic chains (7M and 7D) (Fig. 2-A). 7M and 7D were eluted at the same position as authentic monosialo- and disialo-PA sugar chains, respectively. The ratio of the peak areas of 7N, 7M, and 7D was 55:43:2. A sample of 7M and 7D was treated with sialidase from *Clostridium perfringens* or Newcastle disease virus and analyzed by DEAE-5PW HPLC. Figure 2-B shows that the peak of 7M disappeared completely from the original position after digestion with sialidase from *Clostridium perfringens* and a new peak appeared at the position 7MN. The 7DN peak was similarly obtained from 7D (data not shown). Sialidase from Newcastle disease virus, which is specific to the α2→3 linkage, however, did not result in digestion (Fig. 2-C for 7M, data not shown for 7D). These results suggest that 7M and 7D were of monosialo- and disialo-sugar chains, respectively, and that these sialyl residues were of an α2→6 Gal linkage. To further investigate, 7M and 7D were digested with sialidase from *Clostridium perfringens* and converted to 7MN and 7DN, respectively. All sialic acid released was confirmed to be N-acetylneuraminic acid by the identification method of sialic acid on HPLC (data not shown).

The neutral sugar chains of 7N, 7MN, and 7DN were separated by sequential gel filtration, reversed-phase, and size-fractionation HPLC. Gel filtration HPLC in a GS220 column for the first step of fractionation separated the neutral sugar chain of 7N into two peaks corresponding to 12.4 GU (7N1) and 10.2 GU (7N2) in the ratio of 41:59 (Fig. 3-A). Reversed-phase HPLC in a C18 column for the second step further separated 7N1 into two peaks corresponding to 9.6 GU (7N1A) and 10.1 GU (7N1B) (Fig. 3-B), and 7N2 into 8.1 GU (7N2A) and 9.2 GU (7N2B) (Fig. 3-C). 7MN from the acidic PA-sugar chain was separated by reversed-phase HPLC into three peaks, 7MN1, 7MN2, and 7MN3, corresponding to 8.1, 9.2,
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Fig. 3. Separation of the Neutral PA-Sugar Chains Obtained from PAS-7 Glycoprotein by Gel Filtration HPLC in a GS220 Column (A), by Reversed-phase HPLC in a C18 Column (B-E), and by Size-fractionation HPLC in an Amide-80 Column (F-I).

A, 7N; B, 7N1 in A; C, 7N2 in A; D, asialo PA-sugar chains of 7M; E, asialo PA-sugar chains of 7D; F, 7N1A; G, 7N1B; H, 7N2A; and I, 7N2B.

The closed arrowheads indicate the eluting position of the glucose oligomers indicated by GU (glucose units). The open arrowheads indicate the positions of the standard sugar chains, II, III, and IV, which are described in the Material and Methods section. The peaks eluted at 3-5 min were due to contaminating materials from the reagents.

and 9.6 GU, respectively (Fig. 3-D), while 7DN was also separated into two peaks, 7DN1 and 7DN2, corresponding to 8.1 and 9.6 GU, respectively (Fig. 3-E).

Size-fractionation HPLC in an Amide-80 column for the final step further separated 7N1B into two peaks corresponding to 5.1 GU (7N1B-1) and 7.0 GU (7N1B-2) as shown in Fig. 3-G. The other three fractions, 7N1A, 7N2A, and 7N2B, emerged as single peaks of 7.4, 7.0, and 6.3 GU, respectively (Fig. 3-F, 3-H, and 3-I). 7MN1, 7MN2, 7MN3, 7DN1, and 7DN2 each gave a single peak corresponding to 7.0, 6.3, 7.4, 7.0, and 7.4 GU, respectively, by the size-fractionation HPLC in an Amide-80 column (data not shown).

The carbohydrate compositions of 7N1A, 7N1B-1, 7N1B-2, 7N2A, 7N2B, 7MN1, 7MN2, 7MN3, 7DN1, and 7DN2 are shown in Table 2. The relative proportions of Man and GlcNAc were nearly 3:4 among all the PA-oligosaccharides, suggesting that they were likely to be of the biantennary complex type. Fuc was deficient in PA-oligosaccharides 7N1B-1, 7N1B-2, 7N2A, 7MN1, and 7DN1, while Gal was not detected in 7N1B-1.

Sequential exoglycosidase digestion of the PA-sugar chains

Each PA-sugar chain that had been isolated as a single peak by HPLC in the Amide-80 column was digested with a series of exoglycosidases, and each digest was then analyzed in a TSK gel Amide-80 column. The results described next led us to estimate the sequence of the glycosyl residues and anomeric configuration of individual glycosides.

1) 7N1A. The sugar chain of 7N1A (7.4 GU) was sequentially digested with α-L-fucosidase, β-galactosidase, and β-N-acetylhexosaminidase, which resulted in a new peak at each step. α-Fucosidase digestion altered 7N1A to a sugar chain with 7.0 GU, releasing a Fuc residue. Further digestion with β-galactosidase altered the 7.0 GU sugar chain to 5.1 GU, releasing two Gal residues. β-N-Acetylhexosaminidase digestion altered the 5.1 GU sugar chain to 4.3 GU, eluting at the same position as that of the authentic trimannosyl core sugar chain, Manα1→

Table 2. Carbohydrate Compositions of the Separated N-Linked Sugar Chains of PAS-7 Glycoprotein

<table>
<thead>
<tr>
<th>PA-sugar chains of PAS-7</th>
<th>7N1A</th>
<th>7N1B</th>
<th>7N2A</th>
<th>7N2B</th>
<th>7MN1</th>
<th>7MN2</th>
<th>7MN3</th>
<th>7DN1</th>
<th>7DN2</th>
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<tr>
<td>Fuc</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Man</td>
<td>3.0*</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Gal</td>
<td>1.9</td>
<td>1.7</td>
<td>1.9</td>
<td>1.0</td>
<td>1.8</td>
<td>0.9</td>
<td>1.8</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>3.9</td>
<td>3.6</td>
<td>3.7</td>
<td>3.7</td>
<td>3.8</td>
<td>3.7</td>
<td>3.8</td>
<td>3.7</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Values are given as the molar ratio to mannose of 3.0.
Table 3. Summary of the Sequential Exoglycosidase Digestion of the N-Linked PA-Oligosaccharides of PAS-7 Glycoprotein
Values indicate glucose units by Amide-80 HPLC and the number of monosaccharides released. Numerals in parentheses shows glucose units by reversed-phase HPLC.

| Code of sugar chain | A | Sequential exoglycosidase digestion | B |
|---------------------|--------------------------------|--------------------------------|
|                     | Original chain → α-Fucosidase → β-Galactosidase → β-N-Acetylhexosaminidase | Original chain → α-Mannosidase → β-Galactosidase → β-N-Acetylhexosaminidase |
| 7N1A                | 7.4 (9.6) → 7.0 1 Fuc → 5.1 2 Gal → 4.3* 2 GlcNAc                      | 7.0 → 5.9 1 Man → 4.0 2 Gal → 3.2 2 GlcNAc → 2.0 1 Man |
| 7N1B                |             | 7N1B-1 → 7N1B-2             |                                        |
| 7N1B-1              | 5.1 (7.4) → 7.0 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7N1B-2              | 7.0 (8.1) → 5.9 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7N2A                | 6.3 (9.2) → 7.0 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7N2B                | 7.0 (8.1) → 5.9 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7MN1                | 6.3 (9.2) → 7.0 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7MN2                | 7.4 (9.6) → 7.0 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7MN3                | 7.4 (9.6) → 7.0 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7DN1                | 7.0 (8.1) → 5.9 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |
| 7DN2                | 7.4 (9.6) → 7.0 1 Fuc → 5.1 2 Gal → 4.3 2 GlcNAc                      |                                        |

* 4.3 glucose units was eluted at the same position as that of Manol-6(Manol-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA.

6(Manol-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA, accompanied by the release of two GlcNAc residues. These results show that 7N1A had the sequence of one αFuc, two βGal, and two βGlcNAc bound to trimannosyl core structure.

The susceptibility of the sugar chains from PAS-7 glycoprotein to digestion by the sequential exoglycosidases is summarized in Table 3.

2) 7N2A. 7N2A (7.0 GU) was not digested with α-fucosidase, suggesting that this sugar chain lacked Fuc, which is consistent with results shown in Table 2. The results of the subsequent exoglycosidase digestions show that 7N2A had the sequence of two βGal and two βGlcNAc linked to trimannosyl core structure (Table 3).

3) 7N2B. 7N2B (6.3 GU) was converted to 5.9 GU with α-fucosidase. Subsequent β-galactosidase digestion altered it to 5.1 GU, releasing one Gal residue. The results of the sequential digestion show that 7N2B had the sequence of one αFuc, one βGal, two βGlcNAc, and trimannosyl core structure (Table 3).

4) 7N1B-1. 7N1B-1 (5.1 GU) was not digested with either α-fucosidase or β-galactosidase, showing that this sugar chain lacked Fuc and Gal. β-N-Acetylhexosaminidase digestion converted the 5.1 GU sugar chain to 4.3 GU, indicating the release of two GlcNAc residues. 7N1B-1 was thus estimated to have two βGlcNAc linked to trimannosyl core structure (Table 3).

5) 7N1B-2. 7N1B-2 (7.0 GU) was not digested with α-fucosidase. The results of sequential digestion with β-galactosidase and β-N-acetylhexosaminidase show that 7N1B-2 had the sequence of two βGal and two βGlcNAc linked to a trimannosyl core structure. This sequence is the same as that for 7N2A. 7N2A and 7N1B before separation to 7N1B-1 and 7N1B-2 were eluted with different GU values of 8.1 and 10.1, respectively, from reversed-phase column (Fig. 3-B and 3-C). 7N1B was separated into 7N1B-1 and 7N1B-2 (Fig. 3-G), while 7N2A was not by HPLC with the Amide-80 column (Fig. 3-H), although both showed the same GU value of 7.0 (Table 3). Therefore, the structure of 7N1B-2 is thought to have been different from that of 7N2A. 7N1B-2 was not digested with β-N-acetylhexosaminidase, but α-mannosidase digestion resulted in a new peak corresponding to 5.9 GU, indicating the release of one Man residue. Subsequent digestion with β-galactosidase, β-N-acetylhexosaminidase and α-mannosidase resulted in the release two Gal, two GlcNAc, and one Man residues, respectively (Table 3-B). 7N1B-2 after digestion with β-galactosidase was also digested with α-mannosidase, releasing one Man residue (data not shown). These results show that one of two Man and two Gal residues were positioned at the non-reducing ends and that 7N1B-2 was of a biantennary type with the sequence of two βGal, two βGlcNAc, and one Man forming a branch, terminal one Man linked to trimannosyl core structure.

6) 7MN1, 7MN2, and 7MN3. The asialo sugar chain, 7MN, was separated into three sugar chains, 7MN1 (7.0 GU), 7MN2 (6.3 GU), and 7MN3 (7.4 GU). Among them, only 7MN1 was not digested with α-fucosidase. 7MN1 and the α-fucosidase digests of 7MN2 and 7MN3 showed the same respective digestion patterns by the sequential exoglycosidases as those of 7N2A, 7N2B, and 7N1A (Table 3).

7) 7DN1 and 7DN2. 7DN1 (7.0 GU) and 7DN2 (7.4 GU) were derived from the asialo sugar chain of 7D. 7DN1 was not digested by α-fucosidase. The subsequent digestion of 7DN1 and 7DN2 gave the same
results as those for 7N2A and 7N1A, respectively (Table 3).

Partial acetylation of 7N1B-2

The linkages of one Man and two Gal residues at the non-reducing terminals of 7N1B-2 were confirmed by partial acetylation and re-pyridylation. The reducing terminal oligosaccharide from 7N1B-2 by partial acetylation showed 6.5 GU by reversed-phase HPLC (Fig. 4-B) and was not digested with α-mannosidase (Fig. 4-C). Sequential digestion with β-galactosidase, β-N-acetyhexosaminidase, and α-mannosidase altered the non-reducing ends of 6.5 GU to 5.8, 4.6, and 3.0 GU, respectively (Fig. 4-D, 4-E, and 4-F), with the release of two Gal, two GlcNAc, and one Man residues, respectively. The results of a sugar analysis confirmed that the non-reducing terminal oligosaccharide contained one Man residue by anion-exchange column HPLC (data not shown). These results show that 7N1B-2 was cleaved between Manβ→6Manα arm by partial acetylation, and its proposed structure is as follows:

\[
\begin{align*}
\text{Galβ1→4GlcNAcβ1} & \quad \text{Manα} \\
\text{Galβ1→4GlcNAcβ1} & \quad \text{Manβ1→4GlcNAcβ1} \\
\end{align*}
\]

NMR analysis

The 600-MHz 'H-NMR spectra of the PA-sugar chains are shown in Fig. 5 and the chemical shift values in Table 4. The numbering system used for denoting the glycosyl residues was that of Vliegenthart et al. 20 The presence of the trimannosyl core typical of Asn-linked oligosaccharides was indicated by the Man H-2 atom resonance for all N-linked sugar. This can be deduced from the chemical shifts of H-1 of Man-4 (5.099–5.105 ppm except for 7N1B-2, 5.125 ppm) and of Man-4' (4.913–4.917 ppm) in combination with those of H-2 of Man-3

![Fig. 4. Sequential Exoglycosidase Digestion of 7N1B-2 after Partial Acetylation Analyzed by Reversed-phase HPLC.](image)

![Fig. 5. 600-MHz 'H-NMR Spectra of the Anomeric (left) and Methyl (right) Protons of Oligosaccharides 7N1A, 7N1B-1, 7N1B-2, 7N2A, and 7N2B from PAS-7 Glycoprotein.](image)

7N1B-2 (B) shows the extended spectrum of 7N1B-2 (A). Assignments: 3, Man-3; 4, Man-4; 4', Man-4'; 2, GlcNAc-2; 1, GlcNAc-1; 5, GlcNAc-5; 5', GlcNAc-5'; 6, Gal-6; 6', Gal-6'; Fuc, as shown in Table 4. The probe temperature was 23°C. Wide peak of 4.7–4.9 ppm was from HDO.
(4.217-4.222 ppm except for 7N1B-2 and 7N2B, 4.432 ppm), of Man-4 (4.172-4.185 ppm) and of Man-4' (4.112-4.117 ppm). The conformation freedom of Man-4' occurring of α1-6 linkage is larger than that of Man-4, which is present in a α1-3 linkage. Furthermore, in 7N2B and 7N1A, one Fuc residue linked α1-6 to GlcNAc-1, this being characterized by the chemical shifts of its own structural-reporter groups (CH$_3$, 1.167-1.168 ppm), and by the typical resonance positions of the structural reporters of GlcNAc-2 (Nac, 2.058-2.067 ppm). The chemical shifts for H-1 were identical for GlcNAc-5 and GlcNAc-5' except 7N2B that possess one Gal at the non-reducing ends. The H-1 chemical shift for GlcNAc-5' of 7N2B was altered from 4.546 ppm for GlcNAc-5 to 4.572 ppm when Gal bound to the GlcNAc residue. It was therefore concluded that 7N2B had Gal linked to GlcNAc-5'. The presence of N-acetyllactosamine unit at the terminal ends of 7N1A, 7N1B-2, and 7N2A was indicated that the chemical shifts of H-1 of GlcNAc-5 and -5', Gal-6 and Gal-6' were identical each other. The H-1 chemical shifts for 7N1B-1 that have not Gal bound to the GlcNAc residue were 4.543 ppm for both GlcNAc-5 and -5', while the H-1 chemical shifts for oligosaccharides that possess Gal bound to the GalNAc residues were 4.569-4.578 ppm. 7N1B-2 is considered as being structure with a terminal Man-4' α1-6 linked to β-Man-3. This can be deduced from the chemical shifts of H-2 of Man-3. Compared with the spectra of 7N2A and 7N1B-1, the chemical shifts of H-2 of Man-3 and Man-4' in 7N1B-2 obviously changed. This is characteristic for a terminal α1-6 linked Man group. The proposed structures of the sugar chains from PAS-7 are based on the foregoing results and are summarized in Fig. 6 together with their molar proportions.

Table 4. 600 MHz $^1$H-NMR Chemical Shifts (ppm) to the Structural Reporter Groups of Constituent Monosaccharides of the PAS-7 Glycoprotein Isolated from MFGM

<table>
<thead>
<tr>
<th>Reporter groups</th>
<th>Residue</th>
<th>7N1A</th>
<th>7N1B-1</th>
<th>7N1B-2</th>
<th>7N2A</th>
<th>7N2B</th>
</tr>
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<tbody>
<tr>
<td>H-1</td>
<td>GlcNAc-2</td>
<td>a</td>
<td>a</td>
<td>4.677</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Man-3</td>
<td>a</td>
<td>a</td>
<td>(4.763)</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Man-4</td>
<td>5.101</td>
<td>5.105</td>
<td>5.125</td>
<td>5.099</td>
<td>5.105</td>
</tr>
<tr>
<td></td>
<td>Man-4'</td>
<td>a</td>
<td>a</td>
<td>4.917</td>
<td>4.913</td>
<td>4.913</td>
</tr>
<tr>
<td></td>
<td>GlcNAc-5</td>
<td>4.569</td>
<td>4.543</td>
<td>4.578</td>
<td>4.571</td>
<td>4.546</td>
</tr>
<tr>
<td></td>
<td>GlcNAc-5'</td>
<td>4.569</td>
<td>4.543</td>
<td>4.578</td>
<td>4.571</td>
<td>4.572</td>
</tr>
<tr>
<td></td>
<td>Gal-6</td>
<td>4.458</td>
<td>nd</td>
<td>4.466</td>
<td>4.463</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Gal-6'</td>
<td>4.458</td>
<td>nd</td>
<td>4.466</td>
<td>4.463</td>
<td>4.459</td>
</tr>
<tr>
<td></td>
<td>Fuc</td>
<td>a</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Man-4</td>
<td>4.172</td>
<td>4.177</td>
<td>4.177</td>
<td>4.177</td>
<td>4.185</td>
</tr>
<tr>
<td></td>
<td>Man-4'</td>
<td>4.117</td>
<td>4.116</td>
<td>4.112</td>
<td>4.116</td>
<td>4.115</td>
</tr>
<tr>
<td>NAc</td>
<td>GlcNAc-1</td>
<td>1.958</td>
<td>1.950</td>
<td>1.933</td>
<td>1.931</td>
<td>1.932</td>
</tr>
<tr>
<td></td>
<td>GlcNAc-2</td>
<td>2.063</td>
<td>2.059</td>
<td>2.063</td>
<td>2.058</td>
<td>2.067</td>
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<tr>
<td></td>
<td>GlcNAc-5</td>
<td>2.042</td>
<td>2.050</td>
<td>2.044</td>
<td>2.039</td>
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<tr>
<td></td>
<td>GlcNAc-5'</td>
<td>2.032</td>
<td>2.039</td>
<td>2.039</td>
<td>2.031</td>
<td>2.032</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>Fuc</td>
<td>1.168</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>1.167</td>
</tr>
</tbody>
</table>

○, GlcNAc; ●, Man; ◆, Gal; △, Fuc. a, not detectable due to the limited amount of sample. nd, not detected. GlcNAc-1 is the pyridylaminated residue.

Spectra were measured in $^1$H$_2$O at 23°C (in parentheses, measured at 60°C).
Discussion

The common structural feature among the 10 N-linked sugar chains from PAS-7 glycoprotein is, as
summarized in Fig. 6, of a biantennary complex type linked with one (7N1A, 7N2B, 7MN2, 7MN3, and
7DN2) or no Fuc residue (7N1B-1, 7N1B-2, 7N2A, 7MN1, and 7DN1), and with one (7MN1, 7MN2, and
7MN3) or two NeuAc residues (7DN1 and 7DN2). The one Fuc residue was linked to the GlcNAc residue
at the reducing end through an α1–6 linkage, while the NeuAc residue(s) was linked by an ω2→6
linkage through to the Gal residue at the non-reducing ends. 7N1B-2 has a unique structure with a terminal
Manα1–6 and two N-acetyllactosamine units linked to Manα1–3 arm. This structure was
confirmed in this study by 600-MHz 1H-NMR spectroscopy (Table 4), partial acetylation (Fig. 4), and exoseglycosidase digestion (Table 3). A small amount of
oligosaccharide with such a structure has only been previously reported in γ-glutamyl transpeptidase
from a human hepatoma.32 No oligosaccharide having the structure of 7N1B-1 and 7N1B-2 with NeuAc
was detected in PAS-7. Oligosaccharide with structure of 7N1B-2 with NeuAc, however, has been detected
in normal human liver and hepatoma.32

Although the structures of the sugar chains in PAS-7 were those generally found, the Galβ1→4GlcNAc unit
at the non-reducing ends of the neutral sugar chain (7N1A, 7N1B-2, and 7N2A) has not been found in
most glycoproteins from bovine MFGM: i.e., the GalNAcβ1→4GlcNAc unit was found, instead, at the
non-reducing ends of the glycoproteins from bovine MFGM33 such as CD36 corresponding to PAS-430
and butyrophilin corresponding to PAS-5.33 It has been demonstrated that N-acetylgalactosaminytranserase in the pituitary gland catalyzed the transfer of GalNAc to the GlcNAc residues of the N-

Neutral-sugar chains

(1)  
Galβ1→4GlcNAcβ1→2Manx1
Galβ1→4GlcNAcβ1→2Manx1
  6
  6

(2)  
GlcNAcβ1→2Man1x1
GlcNAcβ1→2Man1x1
  6
  6

(3)  
Galβ1→4GlcNAcβ1→4Man1x1
Galβ1→4GlcNAcβ1→4Man1x1
  6
  6

(4)  
Galβ1→4GlcNAcβ1→2Man1x1
Galβ1→4GlcNAcβ1→2Man1x1
  6
  6

(5)  
Galβ1→4GlcNAcβ1→2Man1x1
GlcNAcβ1→2Man1x1
  6
  6

Fucα1

Fucα1

Fucα1

Fucα1

7MN1  
7N1A  
13.7%
7N1B-1  
3.5%
7N1B-2  
5.3%
7N2A  
16.0%
7N2B  
16.5%

Slalo-sugar chains

(6) ± NeuAcα2→6Galβ1→4GlcNAcβ1→2Manx1
± NeuAcα2→6Galβ1→4GlcNAcβ1→2Manx1
  6
  6

Fucα1

Fucα1

7MN1  
13.8%
7DN1  
0.9%

(7) NeuAcα2→6Galβ1→4GlcNAcβ1→2Manx1
GlcNAcβ1→2Manx1
  6

Fucα1

7MN2  
11.6%

(8) ± NeuAcα2→6Galβ1→4GlcNAcβ1→2Manx1
± NeuAcα2→6Galβ1→4GlcNAcβ1→2Manx1
  6

7MN3  
17.6%
7DN2  
1.1%

Fig. 6. Proposed Structures of the N-Linked PA-Sugar Chains of PAS-7 Glycoprotein.
In 7MN1 and 7MN3, one NeuAc residue is added to either outer Gal, and in 7DN1 and 7DN2, two NeuAc residues are in both outer Gals.
O-linked sugar chain by recognizing the tripeptide sequence, Pro-Xaa-Lys/Arg, located six or nine amino acid residues away from the putative glycosylation sites.36,37 The absence of this tripeptide sequence in PAS-6/7 is likely to have been responsible for the different glycosylation pattern.

Glycoproteins PAS-6 (50 kDa) and PAS-7 (47 kDa) are the major protein constituents of bovine MFGM. As both share a common polypeptide core,13,28 their difference in molecular weight and in affinity to lectin11) is likely to be ascribable only to their different carbohydrate moiety (Table 1). PAS-6/7 consists of 409 amino acid residues deduced from its cDNA10) and has 4 putative glycosylation sites,9 two of which are for N-glycosylation (41st and 209th Asn). The structure of the N-linked sugar chains of PAS-6 has previously been reported by us.16 The quantity of the Man residue was 3 times higher in PAS-6 than in PAS-7 (Table 1). The Manα1-6(Manβ1-3)Manβ1-4GlcNAcβ1-4GlcNAc-structure found in PAS-6,10) was not detected in PAS-7. Hvarregaard et al.11) have recently reported that the sugar chain linked to Asn209 of PAS-6 had a high content of Man, while those linked to Asn41 of both PAS-6 and -7 were slightly different. It is considered that N-linked sugar chains having a similar structure were linked to Asn41 of both PAS-6 and -7, whereas sugar chains with a trimannose core structure were linked to Asn209 of PAS-6, but not to that of PAS-7. The presence of such a structure in PAS-6 could well explain the difference in affinity to Concanavalin A between PAS-6 and -7, which we took advantage of separating PAS-7 from PAS-6.13)

Why, in spite of sharing a common primary structure, does the structure of the N-linked sugar chains of PAS-6 differ from that of PAS-7? One possibility is that a lipid-oligosaccharide intermediate, which is essential for the biosynthesis of an N-linked oligosaccharide, is not transferred to Asn209 of PAS-7 on the pathway for post-transcriptional modification in bovine mammary secretory cells. This is likely to be due to the different localization of these proteins in the Golgi apparatus. It has also been pointed out that the O-glycosylation site in PAS-6 (Ser9) differed from that in PAS-7 (Thr15).11) Two mRNA variants of PAS-6/7 have been found in the mouse mammary gland: the long variant was mammary-specific and lactation-dependent and contained an exon for the Pro-Thr-rich domain, while the short one has been detected in various tissues and decreased during lactation.59) Interestingly, a fully glycosylated product of the long mRNA variant was both N- and O-glycosylated, but a product of the short one was only O-glycosylated.59) PAS-6 and -7 in bovine MFGM are also both N- and O-glycosylated.11) Such findings, however, cannot explain the difference in the N-linked sugar chain between the two glycoproteins. It may be reasonable to consider that this difference is caused by the restricted transfer of lipid-oligosaccharide intermediates to the protein destined to become PAS-7.

Glycoprotein PAS-6/7 is widely distributed in a number of glandular epithelial cells and in several body fluids,8) but has not been detected in bovine plasma and erythrocyte ghosts.9) This suggests that the function of PAS-6/7 is not restricted to the mammary gland and that it must act in processes of a more general nature.11,41) PAS-6 and -7 consist of two EGF-like domains and a tandem repeated structure with a high degree of similarity to the C1 and C2 domains.11) The second EGF-like domain contains the RGD cell adhesion motif for integrins α,β. The C-terminal end of the C2-like domain contains an amphipathic α-helix structure which is bound to phospholipid.9) The O- and N-glycosylation sites are localized at the N-terminal end of PAS-6 and -7, while the trimannose core structure is in the C2 domain of PAS-6. PAS-6 and -7 will make an interesting set for a glycoprotein model to study the biosynthetic mechanism for glycosylation and the functional contribution of the glycosylated moiety.

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References

Structures of the N-Linked Sugar Chains in PAS-7 Glycoprotein


