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REVIEW

Occurrence of reducing terminal *N*-acetylglucosamine 3-sulfate and fucosylated outer chains in acidic N-glycans of porcine zona pellucida glycoproteins

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Structures of acidic N-glycans released from porcine zona pellucida glycoproteins by hydrazinolysis were studied. The results indicated that the acidic glycans are of mono- to tetraantennary complex-type with and without *N*-acetyllactosamine repeating units. Sulfated residues are not only located at the C-6 position of GlcNAc included in the *N*-acetyllactosamine repeating units, but also at the C-6 position of GlcNAc in the non-repeated antennae and at the C-3 position of reducing terminal GlcNAc residue. Analysis of the oligosaccharide fragments released by endo-β-galactosidase digestion and by hydrazine/nitrous acid treatment also revealed that various sulfated and non-sulfated forms of fucosylated structures such as Fucα1 → 2Galβ1 → 4($\pm$ SO_3^- → 6)GlcNAc (type 2H), Galβ1 → 4(Fucα1 → 3)($\pm$ SO_3^- → 6)GlcNAc(Le^x) and Fucα1 → 3 or 4($\pm$ SO_3^- → 6)GlcNAc, are expressed in the repeated outer chain moieties.

***Keywords*: acidic N-glycan, zona pellucida, sulfation, fucosylation**

***Abbreviations*: ZP, zona pellucida; AAL, *Aleuria aurantia* lectin; Con A, concanavalin A; aMan, anhydromannose; PVL, *Psathyrella velutina* lectin; UEA, *Ulex europaeus* agglutinin; SSA, *Sambucus sieboidiana* agglutinin; Le^a, Lewis a; Le^x, Lewis X. Nomenclature for the ZP glycoproteins used here is adapted from that proposed by Harris *et al.* [16] and discussed in greater detail by Hedrick, J. L. [47]. Terms used for pig ZP glycoproteins in this paper and equivalent terms used in past publications from our laboratory and for the mouse ZP glycoproteins are the following: ZPA = 90 K (pig), ZP2 (mouse); ZPB = 55 Kα (pig); ZPC = 55 Kβ (pig), ZP3 (mouse). Subscript OT indicates NaB^{3}H$_4$-reduced glycans.**

Introduction

The Zona pellucida (ZP), an extracellular matrix surrounding eggs, is composed of several glycoproteins and plays important roles in fertilization. ZP glycoproteins are considered to have multiple sperm-binding sites, because it has been shown in mice that ZPC glycoprotein binds to the plasma membrane of acrosome-intact sperm and induces the acrosome reaction, while ZPA glycoprotein binds to acrosome-reacted sperm [1, 2]. Several lines of evidence suggest that sugar moieties of mouse ZPC glycoprotein may work as sperm receptors and an inducer for the acrosome reaction [3–5]. Sulfated and/or fucosylated glycans of ZP glycoproteins have been suggested to play important roles in fertilization from the observation that fucoidin or dextran sulfate inhibited the sperm-egg binding in many species [6]. In pigs, these sulfated polysaccharides inhibited binding of ZP glycoproteins to proacrosin, a zymogen of a serine protease present in the acrosomal vesicle [7, 8], or to sp38, another sperm protein present in the acrosomal region which we have recently reported [9, 10]. Pig acrosin exhibits limited and specific proteolysis of the ZP glycoproteins [11]. Acrosin activity is also inhibited by sulfated polysaccharides [12], which may relate to the interaction of acrosins with the ZP sulfated sugar moieties and to acrosin-assisted sperm binding/penetration mechanisms [13].

Porcine ZP is composed of three kinds of sulfated glycoproteins called ZPA, ZPB and ZPC glycoproteins [14–16], The homologues of these glycoproteins are found in egg

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envelopes in general, including fish, anura and mammals [17]. Because of the convenience for preparing substantial amounts of materials, analysis of the sugar chains has been performed using porcine ZP glycoproteins [18–21]. Our previous studies indicated that neutral glycans are extremely heterogeneous and unique in that considerable amounts of exposed GlcNAc residues are included in both N- and O-glycans [18, 19]. It has also been shown that both acidic N- and O-glycans are highly sulfated [20, 21]. The analysis of acidic N-glycans was performed using a mixture of ZPB and ZPC glycoproteins [20] and demonstrated that sulfate residues are exclusively located at the C-6 position of GlcNAc residues in *N*-acetyllactosamine repeating units. In this study, total ZP glycoproteins including ZPA glycoprotein, in addition to ZPB and ZPC glycoproteins were analysed, because ZPA, B and C glycoproteins inhibited binding of sperm to the ZP in pigs [13] and only ZPA glycoprotein bound to proacrosin and sp38 [9]. Here we provide evidence showing several new structural aspects of sialylated and/or sulfated N-glycans such as the diversity in sulfation site and linkages of sulfation and sialylation, and the presence of various fucosylated structures in the repeated outer chains.

Materials and methods

Chemicals, enzymes and lectins

NaB^3H_4 (340 mCi mmol^{-1}) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 and neuraminidase from *Arthrobacter urefaciens* were purchased from Nacalai Tesque Inc., Kyoto. β-Galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were purified from jack bean meal [22]. β-Galactosidase and β-*N*-acetylhexosaminidase from *Diplococcus pneumoniae* were purchased from Boehringer Mannheim Yamanouchi, Tokyo. Endo-β-galactosidase from *Eschericia freudii* and snail β-mannosidase were purchased from Seikagaku Corporation, Tokyo. Human placental β-*N*-acetylglucosaminidase A, α-fucosidase from bovine epididymis, GlcNAc 3- and 6-sulfates, and GlcN 3- and 6-sulfates were purchased from Sigma Chemical Co., St Louis, MO. α-Fucosidase from *Xanthomonas manihotis* and that from *Arthrobacter sp.* were purchased from New England Biolabs, Beverly, MA and Takarashuzo, Kyoto, respectively. *Aleuria aurantia* lectin (AAL)-Sepharose and *Psathyrella velutina* lectin (PVL)-Affi-Gel 10 were kind gifts from Dr N. Kochibe, Gunma University. Concanavalin A(Con A)-Sepharose was purchased from Pharmacia, Uppsala. *Ulex europaeus*-1 aggluitinin (UEA)-l-agarose and *Sambucus sieboidiana* agglutinin (SSA)-agarose were from Honen Corporation, Tokyo.

Oligosaccharides

Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 4GlcNAcβ1 → 2)Manα1 → 6[Galβ1 → 4GlcNAcβ1 → 4(Galβ1 → 4GlcNAcβ1 → 2)Manα1 → 3]Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ (Gal_4-$GlcNAc_4$-Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$), Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 [Galβ1 → 4GlcNAcβ1 → 4(Galβ1 → 4GlcNAcβ1 → 2)Manα1 → 3]Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ (2,4-branched Gal_3-$GlcNAc_3$-Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$), Galβ1 → 4GlcNAcβ1 → 6(Galβ1 → 4GlcNAcβ1 → 2)Manα1 → 6[Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$ (2,6-branched Gal_3-$GlcNAc_3$-Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$),Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6(Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6) $GlcNAc_{OT}$ (Gal_2-$GlcNAc_2$-Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$) were obtained from recombinant erythropoietin [23]. Other standard oligosaccharides were prepared from the oligosaccharides listed above by exoglycosidase digestion. Anhydromannitol ($aMan_{OT}$), anhydrotalitol, $aMan_{OT}$ 3-sulfate, and $aMan_{OT}$ 6-sulfate were prepared by nitrous acid treatment of GlcN, GalN, GlcN 3-sulfate, and GlcN 6-sulfate followed by NaB^3H_4 reduction, respectively. Galβ1 → 4aMan_{OT} 6-sulfate was prepared from bovine cornea keratan sulfate by hydrazine/nitrous acid treatment followed by NaB^3H_4 reduction, and its desulfated form, Galβ1 → 4aMan_{OT}, was prepared by methanolysis.

Prepartion of ZP glycoproteins and release of N-glycans

ZP glycoproteins were prepared from porcine ovarian oocytes as previously described [24]. The ZP glycoproteins were subjected to hydrazinolysis [25]. The released glycan fraction was reduced with NaB^3H_4 or NaB^2H_4, and acidic glycans which amounted to 67% of the total N-glycans were separated from neutral ones by paper electrophoresis at pH 5.4 as described previously [18].

Glycosidase digestion

Digestion with α-fucosidase from bovine epididymis was performed using 40 mU per 50 µl in 0.1 M sodium acetate buffer, pH 6. Digestions with endo-β-galactosidase [26], β-*N*-acetyl-glucosaminidase A [27] and α-fucosidase from *Arthrobacter sp.* (previously reported as *Corynebacterium*) [28] were performed as described in the cited references. Digestion with α-fucosidase from *Xanthomonas manihotis* was performed according to the manufacturer's protocol and other glycosidase digestions were performed as previously described [29, 30].

Affinity column chromatography on immobilized lectins

Fractionation of glycans by columns of Con A-Sepharose [31], UEA-1-agarose [32] and SSA-agarose [33] were performed as described in the cited references. AAL-Sepharose column chromatography was carried out with some modifications of the previous method [34] as follows. Glycans

were applied to an AAL-Sepharose column and eluted with 10 mM Tris-acetate, pH 7.4, at 4 °C or at room temperature and the bound glycans were eluted with the buffer containing 5 mM Fuc at room temperature. Acidic glycans on a PVL-Affi-Gel 10 column were eluted with five column volumes of 0.1 M Tris-acetate buffer, pH 7.4, and then with another five column volumes of the buffer containing 10 mM GlcNAc at room temperature.

Hydrazine/nitrous acid treatment

The dried glycan samples were heated with anhydrous hydrazine at 105 °C for 48 h in the presence of hydrazine sulfate (2 mg ml^{-1}), and were freed from hydrazine *in vacuo* in the presence of sulfuric acid. Residual hydrazine sulfate was removed by addition of $Ba(OH)_2$ followed by centrifugation and evaporation. Deamination was carried out by incubating the hydrazinolysates in 0.2 ml of 0.4 M acetic acid containing 0.2 M $NaNO_2$ at room temperature for 2 h [35] followed by addition of ethylamine. The solution was passed through a column of Dowex 50 W × 12 (H^+) and the eluate was evaporated under reduced pressure. For detection of the fragments, the dried residue thus obtained was reduced with NaB^3H_4.

Analytical methods

Mild methanolysis for desulfation or mild acid hydrolysis for desialylation was performed by incubating the sample in 0.05 M methanolic hydrogen chloride at 37 °C for 2 h, or in 0.01 N HCl at 100 °C for 15 min. Fractionation of glycans by anion-exchange column chromatography was carried out using a DEAE-5PW column (0.75 × 7.5 cm, Tosoh, Tokyo). The sample was applied to a column equilibrated with 1 mM pyridine acetate buffer, pH 5.4, held for 10 min and eluted by lineally increasing the concentration of the buffer to 150 mM over 45 min and then to 1 M over 100 min. The flow rate was 0.5 ml min^{-1} and 1 ml fractions were collected. Bio-Gel P-4 column chromatography was performed as described [36]. Paper chromatography was carried out by using either of the two solvent systems: solvent I, butanol-1 : ethanol : water (4 : 1 : 1, by volume); solvent II, ethyl acetate : pyridine : acetic acid : water (5 : 5 : 1 : 3, by volume).

Results

Charge heterogeneity of acidic N-glycans released from porcine ZP glycoproteins

The radioactive acidic glycan mixture obtained from ZP glycoproteins was separated from neutral glycans by paper electrophoresis as previously described [18] and subjected to DEAE-HPLC. As shown in Figure 1A, several peaks were obtained, and pooled as fractions **a** to **e**. When the whole acidic fraction was digested with sialidase and analysed by DEAE-HPLC, 29% of glycans were converted to the neutral fraction **AN1**, while others were eluted in the acidic fraction (Figure 1B) which were then converted to neutral ones after desulfation by methanolysis (**AN2** in Figure 1C). The released sulfate residues were detected as methyl sulfate by ion chromatography using a TSK gel IC-Anion-PW column (data not shown). DEAE-HPLC of the fraction **a** after sialidase digestion revealed that this fraction is composed of glycans with either sialic acid or sulfate, because a part of the fraction **a** became neutral after desialylation. In the same manner, it was also evident that fractions **b**, **c** and **d** are composed of glycans containing both sialic acid and sulfate in a molecule in addition to those containing either sialic acid or sulfate residues, and fraction **e** is a mixture of sulfated glycans with and without sialic acid residues. Thus, the acidic glycans are quite heterogeneous in sialylation and sulfation.

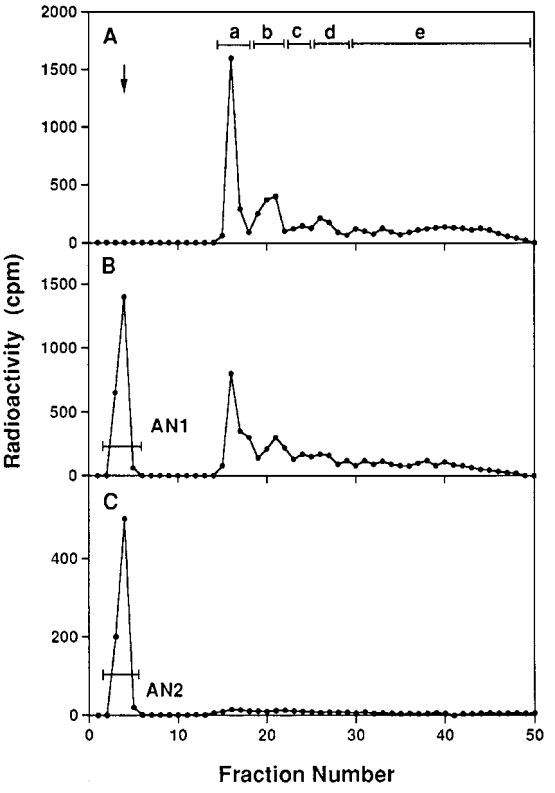


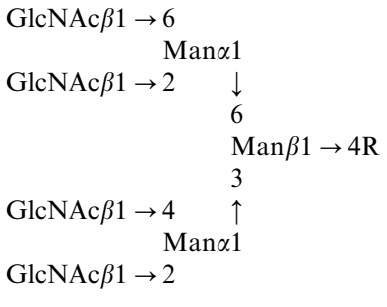
Figure 1. Analysis of acidic glycans by anion-exchange column chromatography. The following samples were fractionated by DEAE-HPLC under the condition as described in 'Materials and Methods'. (**A**) N-linked acidic glycans obtained from porcine ZP glycoproteins by hydrazinolysis, NaB^3H_4 reduction, and paper electrophoresis; (**B**) the glycans in (**A**) digested with sialidase; (**C**) the acidic glycans in (**B**) desulfated by mild methanolysis. An arrow indicates the elution position of neutral glycans.

Analysis of neutral portions of acidic glycans

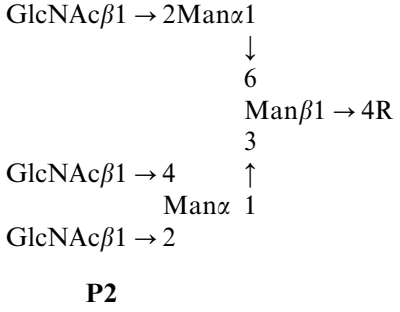
When **AN1** obtained by sialidase digestion of the total acidic glycans (Figure 1B) and **AN2** obtained by the subsequent methanolysis (Figure 1C) were separately applied to

a column of AAL-Sepharose at room temperature, most of them (> 98%) were recovered in the bound fraction. The glycans thus obtained were then separated into the unbound and bound fractions by Con A-Sepharose column chromatography. Percentage molar ratios of the Con A-unbound and the Con A-bound fractions to the total acidic glycans were 14 : 15 for **AN1** and 43 : 28 for **AN2**, respectively.

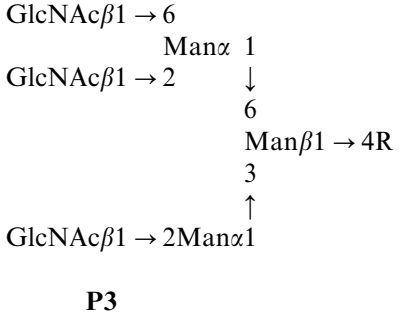
The Con A-unbound fractions of **AN1** (Figure 2A, dotted line) and **AN2** (Figure 2A, solid line) were separated into multiple peaks upon Bio-Gel P-4 column chromatography. Their analysis by sequential glycosidase digestion gave results quite similar to those obtained by our previous analysis of neutral N-glycans of porcine ZP glycoproteins [18], and are therefore described briefly in this paper. Fraction **a** of **AN2** in Figure 2A was sensitive to endo-β-galactosidase, and was then converted into three components **P1**, **P2** and **P3** by diplococcal β-galactosidase (Figure 2B). Fraction **b** in Figure 2A was mostly insensitive to endo-β-galactosidase digestion, and diplococcal β-galactosidase digestion produced **P1**, **P2** and **P3** (data not shown). The structures of **P1**, **P2** and **P3** were identified as follows in the same manner as previously described [18]:



P1



P2



P3

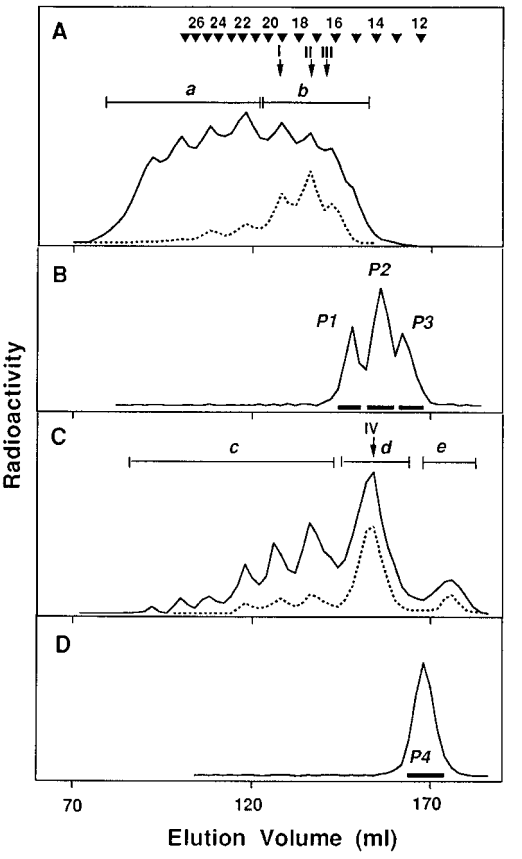


Figure 2. Fractionation of neutral glycans obtained by sialidase digestion and subsequent methanolysis, and their glycosidase digests by Bio-Gel P-4 column chromatography. (**A**) Con A-unbound fractions of **AN1** (dotted line) and **AN2** (solid line); (**B**) the fraction **a** of **AN2** in (**A**) after endo-β-galactosidase digestion followed by diplococcal β-galactosidase digestion; (**C**) Con A-bound fractions of **AN1** (dotted line) and **AN2** (solid line); (**D**) the fraction **c** of **AN2** in (**C**) digested with endo-β-galactosidase and then with diplococcal β-galactosidase. Arrows I, II, III in (**A**) and IV in (**C**) indicate elution positions of authentic Gal_4-$GlcNAc_4$-Man_3-GlcNAc-Fuc-$GlcNaC_{OT}$, 2,4-branched Gal_3-$GlcNAc_3$-Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$, 2,6-branched Gal_3-$GlcNAc_3$-Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$, and Gal_2-$GlcNAc_2$-Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$, respectively. Black arrowheads indicate the elution positions of glucose oligomers, and the numbers indicate the glucose units.

where R represents GlcNAcβ1 → 4(Fucα1 → 6)$GlcNAc_{OT}$. The results indicate that the Con A-unbound fraction is composed of tetraantennary and two isomeric triantennary glycans with (fraction **a**) and without (fraction **b**) *N*-acetyllactosamine repeating units. Sequential glycosidase digestion also revealed that the Con A-unbound fraction of asialo-glycan fraction **AN1** (Figure 2A, dotted line) is similar to **AN2** except that **AN1** contained less amounts of *N*-acetyllactosamine repeating units.

The Con A-bound fraction of **AN1** (Figure 2C, dotted line) and **AN2** (Figure 2C, solid line) also contained multiple components. Fraction **c** of **AN2** in Figure 2C was sensitive to endo-β-galactosidase digestion, and converted to a peak **P4** (Figure 2D) by subsequent diplococcal β-galactosidase

digestion. Fraction **d** in Figure 2D was also converted to the same radioactive component by diplococcal β-galactosidase digestion (data not shown). Peak **P4** was identified as $GlcNAc_2$-Man_3-GlcNAc-Fuc-$GlcNac_{OT}$ as described before [18]. Thus, the Con A-bound fractions contained biantennary glycans with (fraction **c**) and without (fraction **d**) *N*-acetyllactosamine repeating units. Fraction **e** (Figure 2C) is though to contain a monoantennary glycan on the basis of its conversion to fucosylated trimannosyl core with the release of one residue each of Gal and GlcNAc by sequential digestion with diplococcal β-galactosidase and β-*N*-acetylhexosaminidase. The Con A-bound fraction of asialo-glycan fraction **AN1** (Figure 2C, dotted line) was similar to **AN2** except that **AN1** contained smaller numbers of *N*-acetyllactosamine repeating units.

On the basis of these results, the percentage molar ratio of glycans with tetraantennary, 2, 4- and 2, 6-branched triantennary, biantennary and monoantennary structures was calculated to be 14 : 28 : 15 : 38 : 5. The ratio of glycans with and without *N*-acetylelactosamine repeating units was approximately 1 : 1. The presence of comparable amounts of non-repeated sulfated glycans suggests that the sulfate residues are distributed not only to the repeating sequence but also to other parts.

Analysis of sulfation sites

Location of sulfated sugar residues was analysed as follows. The mixture of sulfated glycans (the acidic fraction in Figure 1B) was digested with endo-β-galactosidase. DEAE-HPLC analysis of the digest (Figure 3A) indicated that 54% of the glycans were converted to neutral ones by removal of *N*-acetyllactosamine repeating units. The fraction remained acidic (shown by a bar in Figure 3A) was digested with jack bean β-galactosidase and then with human placental β-*N*-acetylglucosaminidase A, which is known to cleave *N*-acetylglucosaminyl linkages of the $\pm SO_3 \rightarrow 6GlcNAc\beta \rightarrow$ [27]. As shown in Figure 3B, glycans amounting to 36% of the total became neutral, while the residual 10% were eluted at the position corresponding to a monosulfated glycan fraction. When an aliquot of this acidic fraction was desulfated by methanolysis and examined by Bio-Gel P-4 column chromatography, more than 90% of them were eluted at the position of standard Man_3-GlcNAc-Fuc-$GlcNAc_{OT}$. These results suggest that the major sulfated residues are included in the *N*-acetyllactosamine repeating units and in the *N*-acetyllactosamine antennae directly attached to the trimannosyl core, but the minor sulfated residue is in the fucosylated trimannosyl core.

Determination of sulfation site in the trimannosyl core

To identify the sulfated sugar residue in the fucosylated trimannosyl core, the acidic fraction in Figure 3B was analysed as follows. First, the fraction was digested with a mixture of α- and β-mannosidases, producing a radioactive

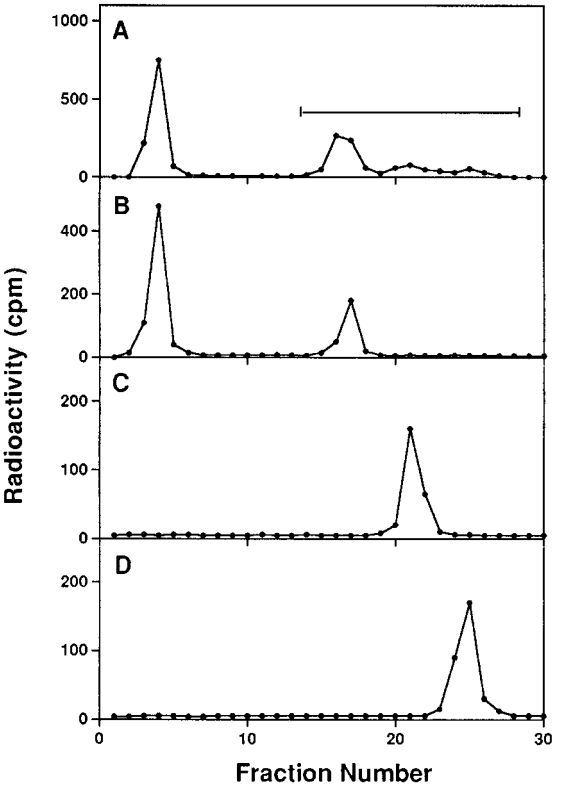


Figure 3. Location of sulfated sugar residues in acid glycans. The following samples were analysed by DEAE-HPLC under the same condition as in Figure 1. (**A**) The sulfated glycans in the acidic fraction (Figure 1B) digested with endo-β-galactosidase; (**B**) the acidic fraction indicated by a bar in (**A**) digested with jack bean β-galactosidase and then with human placental β-*N*-acetylglucosaminidase A; (**C**) the acidic fraction in (**B**) digested with a mixture of α- and β-mannosidases; (**D**) the acidic fraction in (**C**) digested with jack bean β-*N*-acetylhexosaminidase and then α-fucosidase from bovine epididymis.

component bound to a PVL-Affi-Gel 10 colmn which is known to bind GlcNAc-terminated glycans [37]. The glycan recovered from the column was eluted in an acidic fraction upon DEAE-HPLC (Figure 3C) and was supposed to be a sulfated form of fucosyl chitobiitol, $GlcNAc\beta1 \rightarrow 4(Fuc\alpha1 \rightarrow 6)GlcNAc_{OT}$. Actually, its desulfated form was eluted at the position of standard fucosyl chitobiitol, indicating that three mannose residues were removed. The acidic fraction in Figure 3C was then digested with jack bean β-*N*-acetylhexosaminidase. The radioactive product passed through the PVL-column, bound to the AAL-column, and was eluted in an acidic fraction when examined by DEAE-HPLC (data not shown). The product at this stage was supposed to be a sulfated form of $FucGlcNAc_{OT}$ which was then digested with α-fucosidase from bovine epididymis, resulting in the conversion of the radioactive product to an AAL-unbound form by removal of a Fuc residue. The final product thus obtained was eluted at the same position as standard $SO_3^- \rightarrow 3(6)GlcNAc_{OT}$ upon DEAE-HPLC

(Figure 3D), and migrated at the same position as standard $SO_3^- \rightarrow 3GlcNAc_{OT}$ (34 cm from the origin) but not as $SO_3^- \rightarrow 6GlcNAc_{OT}$ (28 cm from the orign) upon paper chromatography using solvent system II for 20 h. These results suggest that the sulfate residue in the trimannosyl core is linked to the C-3 position of the reducing terminal GlcNAc residue.

Analysis of fragments released by endo-β-galactosidase

The total acidic glycans reduced with NaB^2H_4 were digested with endo-β-galactosidase and the released fragments were radiolabelled by NaB^3H_4 reduction. The fragments thus obtained were first subjected to AAL-Sepharose column chromatography at 4 °C, and recovered in an unbound fraction and a bound fraction (8% of the radioactive fragments).

The AAL-bound fraction was separated into a neutral (**n**) and an acidic (**S**) fraction in a ratio 1 : 1 by DEAE-HPLC (Figure 4A). The acidic fraction became neutral by methanolysis (Figure 4B), but not by sialidase digestion, suggesting that the acidic fraction is sulfated. When applied to a Bio-Gel P-4 column, the neutral fraction **n** in Figure 4A was separated into three components **n1**, **n2** and **n3** (Figure 5A) and the desulfated fraction **Sn** in Figure 4B was into two components **Sn1** and **Sn2** (Figure 5C). The components **n1** and **n2** did not bind to an UEA-1-Sepharose column and were resistant to α1-2 fucosidase. But a Fuc residue was removed from each component by α1-3/4 fucosidase (Figure 5B, solid line and dotted line, respectively). On the other hand, component **n3** was resistant to α1-3/4 flucosidase, but was converted to galactitol by α1-2 fucosidase with the release of a Fuc residue (Figure 5B, broken line). The defucosylated forms of components **n1** and **n2** thus obtained were identified as $Gal\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3Gal_{OT}$ and $GlcNAc\beta1 \rightarrow 3Gal_{OT}$, respectively by the same procedure as described previously [18]. These results suggest that the components **n1** and **n2** are $Gal\beta1 \rightarrow 4(Fuc\alpha1 \rightarrow 3)GlcNAc\beta1 \rightarrow 3Gal_{OT}$ and $Fuc\alpha1 \rightarrow 3$ or $4GlcNAc\beta1 \rightarrow 3Gal_{OT}$, respectrively, and the component **n3** is $Fuc\alpha1 \rightarrow 2Gal_{OT}$. The desulfated components **Sn1** and **Sn2** in Figure 5C were shown to have structures identical to components **n1** and **n2** by the same procedure as described above. Thus, the acidic fragments in Figure 4A are suggested to be monosulfated forms of $Gal\beta1 \rightarrow 4(Fuc\alpha1 \rightarrow 3)GlcNAc\beta1 \rightarrow 3Gal_{OT}$ and $Fuc\alpha1 \rightarrow 3$ or $4GlcNAc\beta1 \rightarrow 3Gal_{OT}$.

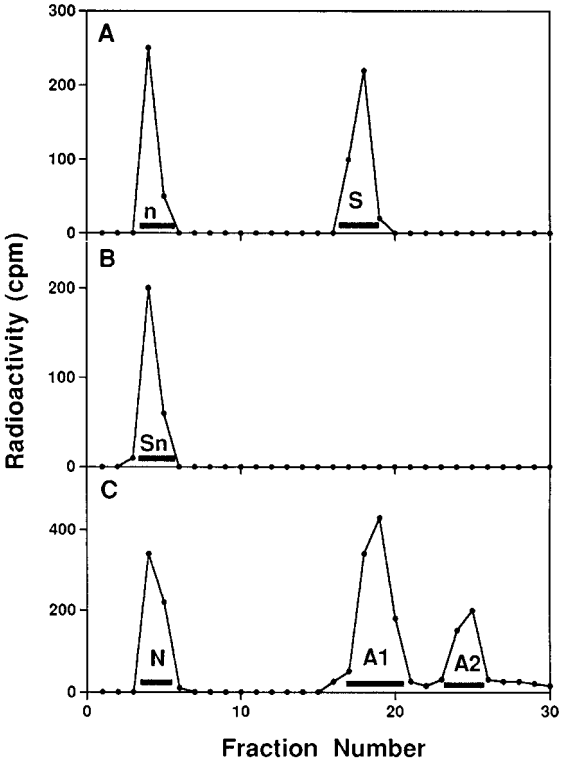


Figure 4. DEAE-HPLC of fragments released by endo-β-galactosidase. Chromatographic conditions are the same as in Figure 1. (**A**) The AAL-bound fraction of fragments released from NaB^2H_4-reduced acidic glycans by endo-β-galactosidase followed by reduction with NaB^3H_4; (**B**) the acidic fraction **S** in (**A**) desulfated by mild methanolysis; (**C**) the AAL-unbound fraction of fragments released by endo-β-galactosidase.

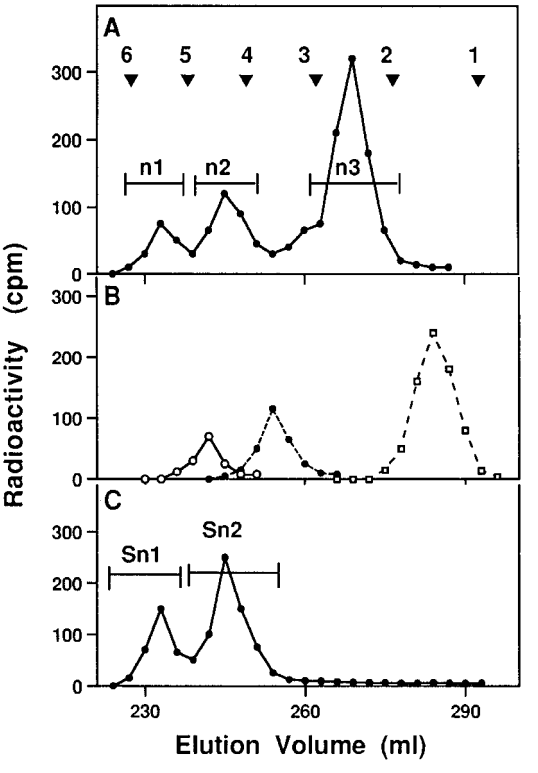


Figure 5. Analysis of fragments ereleased by endo-β-galactosidase by Bio-Gel P-4 column chromatography. (**A**) The AAL-bound neutral fraction **n** in Figure 4A; (**B**) the fractions **n1** (solid line) and **n2** (dotted line) digested with α1-3/4 fucosidase, and **n3** (broken line) digested with α1-2 fucosidase; (**C**) the desulfated AAL-bound fraction **Sn** in Figure 4B. The arrowheads are the same as in Figure 2.

Table 1. Products released from acidic *N*-glycans by endo-β-galactosidase digestion.

Products released	*Ratio (%)*
AAL-bound fraction	
$Gal\beta1 \rightarrow 4(Fuc\alpha1 \rightarrow 3)GlcNAc\beta1 \rightarrow 3Gal_{OT}$	0.4
$Fuc\alpha1 \rightarrow 3/4GlcNAc\beta1 \rightarrow 3Gal_{OT}$	0.7
$Fuc\alpha1 \rightarrow 2Gal_{OT}$	2.9
$Gal\beta1 \rightarrow 4(Fuc\alpha1 \rightarrow 3)(SO_3^- \rightarrow 6)GlcNAc\beta1 \rightarrow 3Gal_{OT}$	1.2
$Fuc\alpha1 \rightarrow 3/4(SO_3^- \rightarrow 6)GlcNAc\beta1 \rightarrow 3Gal_{OT}$	2.8
AAL-unbound fraction	
$GlcNAc\beta1 \rightarrow 3Gal_{OT}$	22.3
$Gal\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3Gal_{OT}$	8.7
$Sia\alpha2 \rightarrow 3Gal\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3Gal_{OT}$	2.5
$Gal\beta1 \rightarrow 4(SO_3^- \rightarrow 6)GlcNAc\beta1 \rightarrow 3Gal_{OT}$	10.0
$SO_3^- \rightarrow 6GlcNAc\beta1 \rightarrow 3Gal_{OT}$	37.5
$Sia\alpha2 \rightarrow 3Gal\beta1 \rightarrow 4(SO_3^- \rightarrow 6)GlcNAc\beta1 \rightarrow 3Gal_{OT}$	11.0

When the AAL-unbound fraction of the fragments released by endo-β-galactosidase was subjected to DEAE-HPLC, a neutral (**N**) and two acidic (**A1** and **A2**) fractions were obtained (Figure 4C). In agreement with the previous study [20], their analysis revealed that fraction **N** contained $\pm\, Gal\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3Gal_{OT}$, and fractions **A1** and **A2** contained $\pm\, Gal\beta1 \rightarrow 4(SO_3^- \rightarrow 6)GlcNAc\beta1 \rightarrow 3Gal_{OT}$ and $Sia\alpha2 \rightarrow 3Gal\beta1 \rightarrow 4(SO_3^- \rightarrow 6)GlcNAc\beta1 \rightarrow 3Gal_{OT}$, respectively (data not shown).

The structures of the fragments released by endo-β-galactosidase digestion and their molar ratio are shown in Table 1. From the known substrate specificity of endo-β-galactosidase, the fragments, except for $\pm\, SO_3^- \rightarrow 6GlcNAc\beta1 \rightarrow 3Gal_{OT}$, are considered to be derived from the reducing termini of the *N*-acetyllactosamine repeating units. Therefore, it is calculated that the ratio of fucosylated and non-fucosylated non-reducing terminal fragments is 1 : 4.

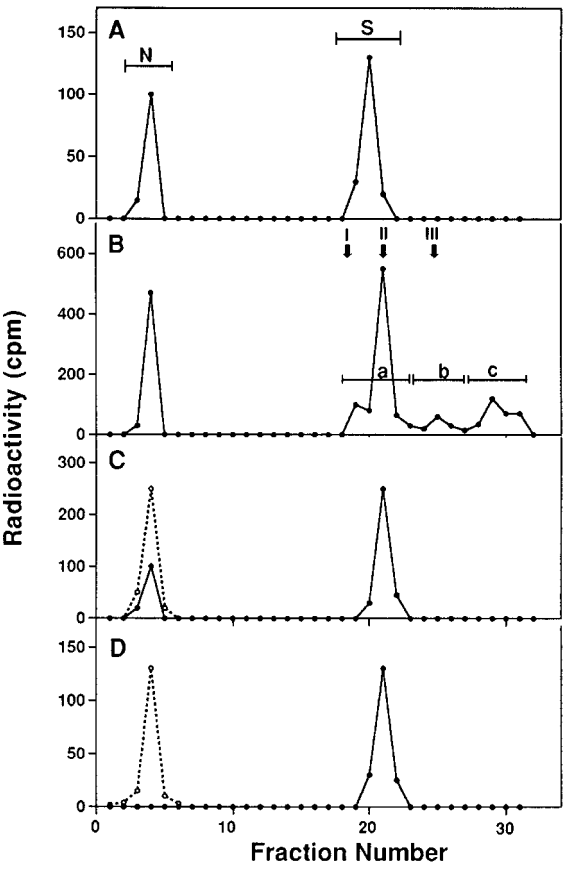


Figure 6. Analysis of deamination products by DEAE-HPLC. Chromatographic conditions are the same as in Figure 1. (**A**) AAL-bound fraction of fragments obtained by hydrazine/nitrous acid treatment of acidic glycans of NaB^2H_4-reduced acidic glycans followed by NaB^3H_4 reduction; (**B**) AAL-unbound fraction of fragments obtained by hydrazine/nitrous acid treatment; (**C**) the fraction **a** in (**B**) desialylated by mild acid hydrolysis (solid line) and the acidic fraction in (**C**) desulfated by mild methanolysis (dotted line); (**D**) the fraction **c** in (**B**) desialylated by mild acid hydrolysis (solid line) followed by mild methanolysis (dotted line). Arrows I, II and III in (**B**) are elution positions of standard $Sia\alpha2 \rightarrow 3Gal\beta1 \rightarrow 4aMan_{OT}$, $Gal\beta1 \rightarrow 4(SO_3^- \rightarrow 6)aMan_{OT}$ and $SO_3^- \rightarrow 6aMan_{OT}$, respectively.

Analysis of deamination products

To confirm the presence of fucosylated outer chains and to further analyse the outer chain moities, including the non-repeated *N*-acetyllactosamine antennae, the NaB^2H_4-reduced acidic glycans were subjected to hydrazine/nitrous acid treatment to cleave *N*-acetylglucosaminyl linkages and the resulting fragments were labelled by NaB^3H_4 reduction. The radioactive products thus obtained were separated into an unbound fraction and a bound fraction (7% of the radioactive products) by AAL-Sepharose column chromatography at 4 °C.

The AAL-bound deamination product was separated into a neutral (**N**) and an acidic (**S**) fraction in a ratio of 2 : 3 by DEAE-HPLC (Figure 6A). Then each of the fractions **N** and **S** were desulfated by methanolysis and subjected to Bio-Gel P-4 column chromatography which produced two fractions with sizes of 3.5 and 2.5 glucose units (Figure 7A and B, respectively). A part of the 3.5 glucose unit fraction was sensitive to α1-2 fucosidase (Figure 7C) and the residual was sensitive to α1-3/4 fucosidase (Figure 7D), and the resulting products were eluted at the position of $Gal\beta1 \rightarrow 4aMan_{OT}$. These results suggest the 3.5 glucose unit fraction contained both glycans with Type 2H-antigenic and Le^X structures, $Fuc\alpha1 \rightarrow 2Gal\beta1 \rightarrow 4aMan_{OT}$ and $Gal\beta1 \rightarrow 4(Fuc\alpha1 \rightarrow 3)aMan_{OT}$. As the 2.5 glucose unit fraction in Figure 7A and B was sensitive to α1-3/4 fucosidase and the product was eluted at the position of $aMan_{OT}$, its structure was suggested to be $Fuc\alpha1 \rightarrow 3$ or $4aMan_{OT}$. Thus, the results were compatible with those obtained from the analysis of the AAL-bound fraction of the oligosaccharides released by endo-β-galactosidase digestion.

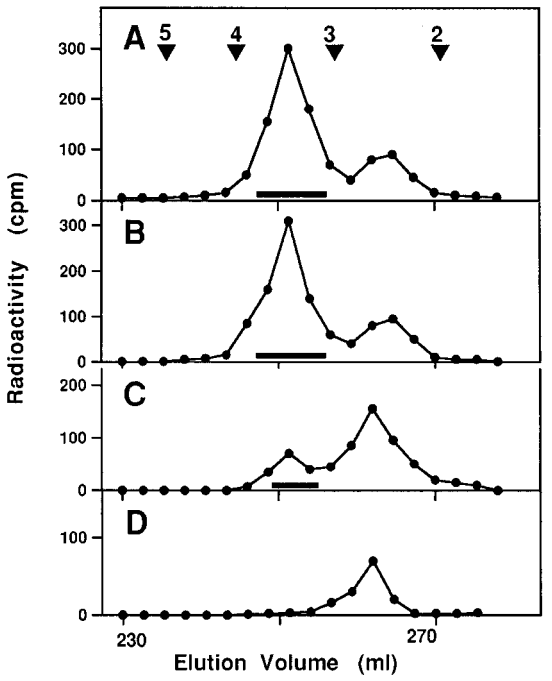


Figure 7. Bio-Gel P-4 column chromatography of deamination products. (**A**) AAL-bound neutral deamination products in Figure 6A; (**B**) AAL-bound acidic products in Figure 6A desulfated by mild methanolysis; (**C**) the fraction with 3.5 glucose units in (**A**) and (**B**) digested with α1-2 fucosidase; (**D**) the resistant fraction indicated by a bar in (**C**) digested with α1-3/4 fucosidase. The arrowheads are the same as in Figure 2.

The AAL-unbound deamination product was separated into a neutral and three acidic fractions **a**, **b** and **c** by DEAE-HPLC (Figure 6B). Fraction **a** is a mixture of sialylated and sulfated fragments, because a part of this fraction became neutral after desialylation by mild acid hydrolysis (Figure 6C, solid line), and the residual also became neutral after desulfation by subsequent methanolysis (Figure 6C, dotted line). When fraction **a** in Figure 6B was subjected to paper chromatography for 22 h using solvent system II, it separated into a major sulfated fragment with an identical mobility to standard Galβ1 → 4(SO_3^- → 6)aMan$_{OT}$ and two sialylated minor fragments which migrated at the positions of standard Siaα2 → 6Galβ1 → 4aMan$_{OT}$ and Siaα2 → 3Galβ1 → 4aMan$_{OT}$ prepared from fetuin with relative mobilities to Galβ1 → 4(SO_3^- → 6)aMan$_{OT}$ of 0.48 and 0.59, respectively. Their identity was confirmed by Smith degradation which resulted in the production of aMan$_{OT}$ from the α2 → 6 sialyl isomer and Galβ1 → 4aMan$_{OT}$ from the α2 → 3 sialyl isomer, respectively. Therefore, it is suggested that fraction **a** is composed of Siaα2 → 6Galβ1 → 4aMan$_{OT}$, Siaα2 → 3Galβ1 → 4aMan$_{OT}$ and Galβ1 → 4(SO_3^- → 6)aMan$_{OT}$. Fraction **b** was identified as SO_3^- → 6aMan$_{OT}$ because of its elution position upon DEAE-HPLC (Figure 6B) and co-migration with the standard on paper chromatography (data not shown). Fraction **c** contained a sulfated fragment with a sialic acid, because it was converted to the monosulfated fragment after desialylation by mild acid hydrolysis (Figure 6D, solid line) and became neutral by subsequent methanolysis (Figure 6D, dotted line). The neutral and the monosulfated fragments thus obtained had the same mobilities as standard Galβ1 → 4aMan$_{OT}$ and Galβ1 → 4(SO_3^- → 6)aMan$_{OT}$, respectively by paper chromatography (not shown). Since Smith degradation of fraction **c** produced Galβ1 → 4(SO_3^- → 6)aMan$_{OT}$ and SO_3^- → 6aMan$_{OT}$ in a ratio 2:1, the presence of two isomers of Siaα2 → 3Galβ1 → 4(SO_3^- → 6)aMan$_{OT}$ and Siaα2 → 6Galβ1 → 4(SO_3^- → 6)aMan$_{OT}$ were suggested. Thus, it should be noted that Siaα2 → 6Galβ1 → 4($\pm$ SO_3^- → 6)aMan$_{OT}$ were detected in addition to their Siaα2 → 3 isomers.

Structure of acidic glycans

Taken together, the structure of the acidic N-glycans is proposed to be that shown in Figure 8. This proposal includes the following structural features. Sulfate residues are mainly linked to the C-6 position of GlcNAc residues in the *N*-acetyllactosamine repeating units and in the antennary portion directly linked to the trimannosyl core. Sulfation also occurs to a small extent at the C-3 position of the reducing terminal GlcNAc residue.

Some of GlcNAc residues are exposed at the nonreducing termini and partly sulfated as shown by the results of hydrazine/nitrous acid treatment. Fucose residues are linked not only to the reducing terminal GlcNAc residue but also, to a small extent, to the outer chain moieties producing Type 2H-antigenic and Le^x structures with and without sulfation. Only the Siaα2 → 3Gal linkage is expressed on the repeated *N*-acetyllactosamine units, while both Siaα2 → 6Gal and Siaα2 → 3Gal linkages are on the non-repeated *N*-acetyllactosamine antennae. This is based on the analysis of fragments released by endo-β-galactosidase and hydrazine/nitrous acid treatment. The presence of both sialyl linkages in the non-repeated antennae was further confirmed as follows. The NaB^3H_4-labelled acidic glycans were digested with endo-β-galactosidase to remove *N*-acetyllactosamine repeating units, and the glycans that still remained acidic were recovered using a DEAE-column. The acidic glycans thus obtained were applied to a column of SSA-agarose which binds glycans with the Neu5Acα2 → 6Galβ1 → 4group [33], and resulted in the separation of the unbound and bound fractions in a ratio 7:3. Both fractions actually contained sialidase-sensitive glycans.

Discussion

The glycans of porcine ZP glycoproteins elucidated here are classified into two groups in terms of their acidic nature; glycans containing only sialic acid residues as negative charged residues (29%) and sulfated glycans with and without sialic acids (71%). Their neutral portions are similar in

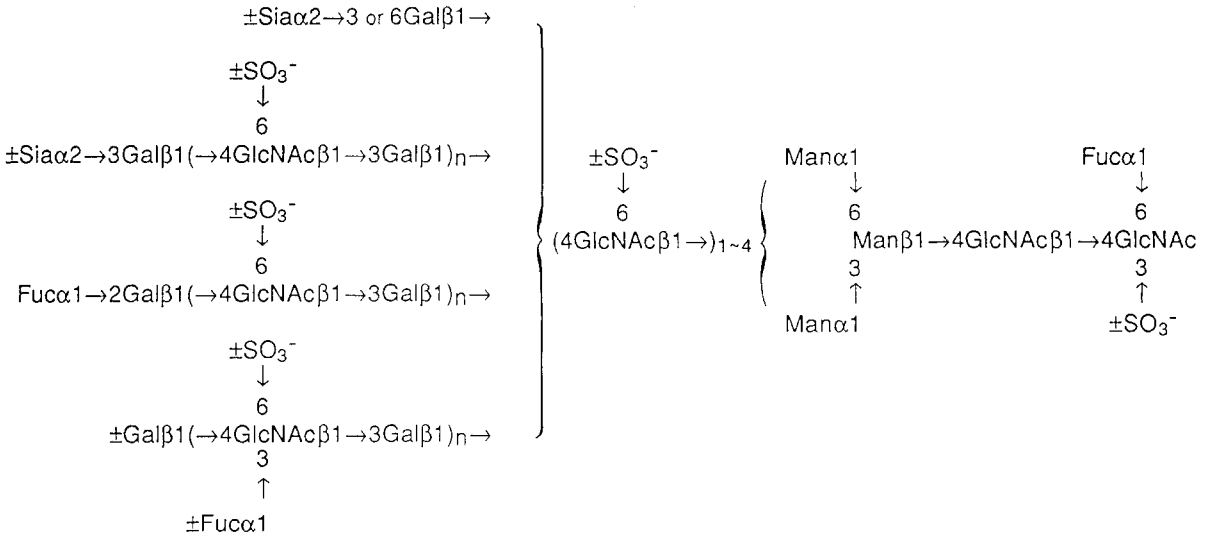


Figure 8. Proposed structures of N-linked acidic glycans included in porcine ZP glycoproteins.

that both groups of glycans have a series of complex-type structures, but differ in that the sialic acid-containing glycans include smaller number of *N*-acetyllactosamine repeating units than the sulfated glycans. In addition, several new structural aspects of acidic N-glycans were revealed as below.

The first is variations found in the linkage and the site of sulfation. In addition to the $SO_3^- \rightarrow 6$GlcNAc residues located in the *N*-acetyllactosamine repeating units [20], we found that considerable amounts of the $SO_3^- \rightarrow 6$GlcNAc residues are attached to the α-mannose residues of the fucosylated trimannosyl core. Occurrence of the sulfated GlcNAc in the non-repeated antennae is consistent with the present result that approximately 40% of the sulfated glycans do not contain the *N*-acetyllactosamine repeating units. We also found that sulfation occurs to a small but significant extent at the C-3 position of the reducing terminal GlcNAc residue. Sulfation of the reducing terminal GlcNAc residue of N-glycan has been suggested to occur in chicken adipose lipoprotein lipase, but the sulfate was proposed to be linked to the C-6 position of GlcNAc [38]. Considering the observation that ZPB retains sperm binding activity after endo-β-galactosidase digestion [39], it is suggested that oligosaccharides without *N*-acetyllactosamine repeating units or the non-repeated portion of the glycans may partly play some role in sperm-egg binding. In this context, the structural feature that sulfated GlcNAc residues are located at the antennae and at the reducing termini is of interest.

Secondly, we not only confirmed the presence of the Siaα2 → 3Gal linkage in the *N*-acetyllactosamine repeating units [20], but also found that two isomeric sialyl linkages are included in the non-repeated antennae as Siaα2 → 3 and 6Galβ1 → 4(± SO_3^- → 6)GlcNAc. As compared to the Siaα2 → 3Galβ1 → 4(SO_3^- → 6)GlcNAc, the Siaα2 → 6Galβ1 → 4(SO_3^- → 6)GlcNAc sequence is only rarely found in a few glycans such as N-glycans of porcine thyroglobulin [40] and keratan sulfate chains of bovine articular cartilage [41].

Finally, it was found that sulfated and non-sulfated forms of type 2H-antigenic determinants, Fucα1 → 2Galβ1 → 4(± SO_3^- → 6)GlcNAc, and Le^x determinants, Galβ1 → 4(Fucα1 → 3)(± SO_3^- → 6)GlcNAc, are included in the *N*-acetyllactosamine repeating units of acidic N-glycans. We also found the agalactosylated forms of sulfated and non-sulfated Le^x structures, ± SO_3^- → 6(Fucα1 → 3 or 4)GlcNAcβ1 → 3Gal, in the fragments released by *E. feundii* endo-β-galactosidase digestion. Since this enzyme does not cleave β-galactosyl linkage in the sequence, → GlcNAcβ1 → 3Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → 3Galβ1 →, the fragments do not seem to be derived from the internal region of the repeating units, but from the non-reducing terminal region. This was also supported by the result that the Fuc-containing fragments, ± SO_3^- → 6(Fucα1 → 3)aMan, were obtained by hydrazine/nitrous acid treatment.

Occurrence of fucosylated oligosaccharide moieties with and without sulfation in N-glycans of porcine ZP glycoproteins is particularly of interest in the light of the proposed roles of fucosylated and/or sulfated glycans in fertilization. It has been shown that fucoidin, a sulfated fucose polymer, inhibits binding of boar spermatozoa to the oocyte ZP [42] and boar sperm contain lectin-like molecules which bind fucosylated neoglycoproteins [43]. The ZP-binding activities of boar sperm proteins, sp38 [9] and a 15 kDa-glycoprotein [44] are inhibited by fucoidin or dextran sulfate. It should be noted that sp38 and proacrosin of boar sperm bind to ZPA [9], which has been proposed as a secondary receptor for acrosome-reacted sperm [2]. The fucose binding activity of proacrosin [43] and the proteolytic activity of acrosin [12] are also inhibited by sulfated polysaccharides.

Thus, accumulating evidence suggests that sulfated and/or fucosylated glycans of ZP glycoproteins play important roles in the binding of boar sperm to eggs and penetration of the sperm. Structural information obtained in this study will help us identify the physiological carbohydrate ligands serving as sperm receptors.

Variations found in the linkage and the location of sulfation, the presence of the Siaα2 → 6Gal linkage, and fucosylation of the *N*-acetyllactosamine repeating units have not been described in the previous study which analysed carbohydrate structures of a mixture of ZPB and ZPC glycoproteins of porcine ZP [20]. This discrepancy could be derived from the difference of materials, since we used the total ZP glycoproteins including ZPA, ZPB and ZPC glycoprotein species to obtain the whole structural features of sugar chains. If that were true, some of the structural moieties newly found in the present study might be expressed on the ZPA glycoprotein species which accounts for 23% (based on the carbohydrate content) or 33% (based on the protein content) of the total ZP glycoproteins [46]. It will be important to examine this possibility in future because such information will be helpful in explaining the discrete biological function of each ZP glycoprotein species.

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