Thompson, A. R., & Enfield, D. L. *Biochemistry 17*, 4304–4311.
Tillet, W. S., & Francis, T., Jr. (1930) *J. Exp. Med. 52*, 561–571.
Volanakis, J. E., & Kaplan, M. H. (1971) *Proc. Soc. Exp. Biol. Med. 136*, 612–614.
Volanakis, J. E., & Wirtz, K. W. A. (1979) *Nature 281*, 155–157.

Comparison of N-Glycosides of Fetuins from Different Species and Human α_2-HS-Glycoprotein

Tetsuo Hayase,[†,§] Kevin G. Rice,[†,∥] Katarzyna M. Dziegielewska,[⊥] Mark Kuhlenschmidt,[#] Thomas Reilly,[#] and Yuan Chuan Lee*[,‡]

Department of Biology, Johns Hopkins University, Baltimore, Maryland, Research Center, Mitsubishi Kasei Corporation, Yokohama, Japan, Clinical Neurological Sciences, University of Southampton, Southampton, United Kingdom, and Department of Veterinary Pathobiology, University of Illinois, Urbana, Illinois

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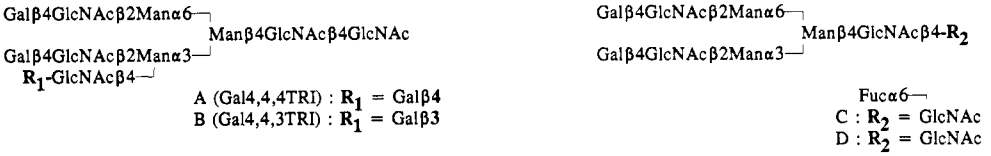
ABSTRACT: Complex type N-glycosides of commercial bovine fetuin preparations from pooled fetal calf serum have been shown to contain comparable amounts of Gal4,4,4TRI (see structure A below) and Gal4,4,3TRI (structure B) as major asialo-structures. To investigate whether there is a clear genetic specificity for synthesis of these oligosaccharides, N-glycosides from two preparations of bovine fetuin, each from a single calf, were examined. Both of these structures were present in each calf, and there was only a subtle quantitative difference in the ratio of these two structures between the calves. Thus, a specific galactosyltransferase, presumably required for the biosynthesis of the Gal4,4,3TRI structure, may exist in both of these individual calves. Comparison of fetuin N-glycosides was also extended to sheep, pig, and human α_2-HS-glycoprotein, the human counterpart of bovine fetuin, using high-pH anion-exchange chromatography of the reducing oligosaccharides as well as HPLC of their pyridinylamino derivatives. The N-glycosides of ovine fetuin also have both Gal4,4,4TRI and Gal4,4,3TRI structures in a ratio similar to that of bovine fetuin. However, the major N-glycoside of porcine fetuin is of a fucosyl biantennary complex type structure (structure C below) and human α_2-HS-glycoprotein has an N-glycoside which is almost exclusively a nonfucosylated biantennary structure (structure D). This species-specific presence of N-glycosides of fetuins and comparison with N-glycosides of other glycoproteins suggest that the polypeptide sequence of a glycoprotein may affect its N-glycan structure by regulating the activity of specific glycosyltransferases.



Fetuin is a fetal glycoprotein, expression of which is apparently regulated developmentally (Spiro, 1960; Dziegielewska et al., 1980a,b). The carbohydrate chains of bovine fetuin have been intensively studied (Nilsson et al., 1979; Townsend et al., 1986, 1989; Takasaki & Kobata, 1986; Green et al., 1988). Bovine fetuin was found to contain two different triantennary complex type oligosaccharides, Gal4,4,3TRI[1] and Gal4,4,4TRI (Townsend et al., 1986, Takasaki & Kobata, 1986; see structures in the abstract).

It has been shown previously that several commercial preparations of bovine fetuin contain different ratios of these two structures (Townsend et al., 1989). Since the commercial preparations are usually from pooled sources, each of these two different structures may be derived from individual calves. In this study, we obtained two preparations of bovine fetuin, each from a single calf, and examined the ratio of Gal4,4,4TRI and Gal4,4,3TRI structures in these preparations as compared to the ratio in a commercial preparation which was made by the same method.

Recently, the amino acid sequences of fetuins from sheep and pig have been elucidated (Brown et al., 1992). These fetuins as well as α_2-HS-glycoprotein, human fetuin, have close homology in amino acid sequence, especially around the N-glycosylation sites, with bovine fetuin. Therefore, we also compared the carbohydrate structures of fetuins from different species, by high-pH anion-exchange chromatography (HPAEC) of the released N-linked oligosaccharides and by HPLC of pyridinyl-2-amino derivatives of the oligosaccharides (PA-oligosaccharides; Hase et al., 1979).

* Author to whom correspondence should be addressed.
† Johns Hopkins University.
§ Mitsubishi Kasei Corp.
∥ Present address: Department of Pharmaceutics and Pharmaceutical Chemistry, The Ohio State University, Columbus, Ohio.
⊥ University of Southampton.
University of Illinois.

[1] Abbreviations: TRI, triantennary complex type oligosaccharide; HPAEC, high-pH anion-exchange chromatography; PA, pyridinylamino; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; CHO, Chinese hamster ovary; BHK, baby hamster kidney; Gn-T, *N*-acetylglucosaminyltransferase.

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Experimental Procedures

Materials. Two bovine fetuin preparations were isolated from two individual newborn Holstein calves by Spiro's method (Spiro, 1960). A commercial preparation of bovine fetuin, which was also isolated by the Spiro method, was obtained from GIBCO–BRL (Gaithersburg, MD). Ovine and porcine fetuins were isolated by the method of Marti et al. (1973), and their purity was examined as described previously (Brown et al., 1992). Human α_2-HS-glycoprotein was a gift from Dr. Müller-Esterl of University of Munich (Kellermann et al., 1989). TPCK-treated trypsin was from Worthington Biochemical Corp. (Freehold, NJ). Glycopeptidase F and α-fucosidase (bovine kidney) were from Boehringer Mannheim (Indianapolis, IN). *Arthrobacter ureafaciens* neuraminidase was kindly donated by Dr. Tsukada, Kyoto Research Laboratory (Uji, Japan). Reference asialooligosaccharides of Gal4,4,4TRI and Gal4,4,3TRI structure were prepared from the bovine fetuin glycopeptides (Rice et al., 1990), and the biantennary complex type oligosaccharide was from bovine fibrinogen. Standards of pyridinylamino derivatives of fucosylated and nonfucosylated biantennary complex type oligosaccharides were purchased from Takara Shuzo (Kyoto, Japan).

Monosaccharide Analysis. Determination of carbohydrate composition of fetuins was performed basically as described previously (Hardy et al., 1988). Briefly, acid hydrolysis of the samples was carried out in 2 M TFA at 100 °C for 4 h for neutral sugars and in 4 M HCl at 100 °C for 5 h for amino sugars. The dried hydrolysates were dissolved in water and analyzed by HPAEC under the conditions described later.

Preparation of Oligosaccharides from Fetuins. N-Linked oligosaccharides were prepared from two individual preparations of bovine fetuin isolated from a single neonatal Holstein calf and from a commercial preparation as follows. Each bovine fetuin was denatured in 8 M guanidine hydrochloride, reduced with dithiothreitol, alkylated with iodoacetic acid, and dialyzed (Rice et al., 1990). The reduced and alkylated fetuin was digested with trypsin and separated by a Sephadex G-50 gel filtration column, 2.5 × 100 cm, eluted with 0.5% pyridinium acetate buffer. The glycopeptide fractions were monitored by the modified phenol–sulfuric acid method (McKelvy & Lee, 1969) and pooled and dried. About 50 nmol of glycopeptides (based on the phenol–sulfuric acid assay) was dissolved in 10–50 μL of 10 mM ammonium acetate buffer, pH 8.5, and incubated with 0.2 unit[2] of glycopeptidase F at 37 °C for 20 h. The digest was evaporated and treated with 0.1 M acetic acid in order to accelerate the hydrolysis of glycosylamines to unmask the aldehydic groups. The oligosaccharides were dissolved in a small amount of water and 0.1–1 nmol of each was used for analysis by HPAEC.

N-Linked oligosaccharides of ovine and porcine fetuins and human α_2-HS-glycoprotein were prepared by the same method as above and also by the direct method described below. A sample of fetuin (1–5 mg) was dissolved in 0.1 mL of 10 mM ammonium acetate buffer, pH 8.4, with 0.2% 2-mercaptoethanol, and digested with 1 unit of glycopeptidase F by incubation at 37 °C for 2 days. After ammonium acetate and 2-mercaptoethanol were removed by repeated evaporation using a Speed Vac concentrator with water added each time, a portion of the reaction product was directly analyzed by HPAEC.

[2] One unit of this enzyme defined by Boehringer Mannheim (the definition used here) is equal to 1 milliunit on the basis of Tarentino's definition which conforms to IBU standard (Tarentino et al., 1985).

Desialylation of the Oligosaccharides. The released oligosaccharides were also analyzed as asialooligosaccharides after desialylation with *A. ureafaciens* neuraminidase. Sialyloligosaccharides (10–25 nmol) were dissolved in 0.1 mL of 100 mM ammonium acetate buffer, pH 5.5, and incubated with 5 milliunits of the enzyme at 37 °C overnight. The reaction mixture containing the asialooligosaccharides was analyzed by HPAEC after ammonium acetate was removed by repeated evaporation.

HPAEC. A Dionex Bio-LC system equipped with a pulsed amperometric detector (PAD-II) was used for HPAEC (Lee, 1990) of sialyloligosaccharides, desialylated oligosaccharides, and monosaccharides. The eluant used for analysis of sialyloligosaccharides was 0.1 M sodium hydroxide with a 0–0.3 M gradient of sodium acetate concentration in 50 min. For asialooligosaccharide analysis, 0.15 M sodium hydroxide with a 0–0.10 M gradient of sodium acetate in 30 min was used. Isocratic elution with 16 mM sodium hydroxide was used for determination of the monosaccharide composition of the acid hydrolysates (Hardy et al., 1988). In all of the above HPAEC, the flow rate was 1.0 mL/min and elution was at ambient temperature.

PA Analysis. Asialooligosaccharides from porcine fetuin and human α_2-HS-glycoprotein were derivatized with 2-aminopyridine (Kondo et al., 1990). After purification by gel filtration on a Sephadex G-15 column, pyridinylamino derivatives of the asialooligosaccharides (PA-oligosaccharides) were analyzed by reverse-phase HPLC and by amide–silica gel HPLC (Tomiya et al., 1988). Both HPLC analyses were performed on a gradient HPLC system (Gilson, Middleton, WI) with a fluorescence detector (Perkin Elmer LS-40; Norwalk, CT). For reverse-phase HPLC, the column used was a Shimadzu CLC-ODS, 6 × 150 mm (Kyoto, Japan) and the eluant was 10 mM sodium phosphate buffer, pH 4, with a gradient of 1-butanol 0.1–0.25% in 60 min. The flow rate was 1.0 mL/min, and the column temperature was 55 °C. For amide–silica gel HPLC, a Tosoh Amide-80 column, 4.6 × 250 mm (Tokyo, Japan) was used and the eluant was triethylamine–acetic acid buffer, pH 7.3, with a gradient of 65–50% acetonitrile in 50 min. The flow rate was 1.0 mL/min, and the column temperature was 40 °C. For both HPLC experiments, the effluent was monitored by fluorescence, using an excitation wavelength of 320 nm and an emission of 400 nm.

α-Fucosidase Treatment of the Oligosaccharides. Asialooligosaccharides from porcine fetuin and their PA derivatives were dissolved in 50 mM ammonium acetate buffer, pH 5.4, and treated with α-fucosidase from bovine kidney, using 20 milliunits of the enzyme for 1 nmol of oligosaccharides. The reaction mixtures were incubated at 37 °C for 50 min and analyzed by HPAEC or HPLC after evaporation.

Fast Atom Bombardment Mass Spectrometry (FAB-MS) of PA-Oligosaccharides. PA-oligosaccharides from porcine fetuin were analyzed by FAB-MS in a positive-ion detection mode. A few nanomoles of the sample was dissolved in dimethyl sulfoxide and analyzed on a JMS-HX110 mass spectrometer (JEOL, Akishima, Japan) with an acceleration voltage of 10 kV. Glycerol was used as a matrix and dithioerythritol was added to enhance PA-oligosaccharides affinity to the matrix.

Results

Release of N-Glycosides from Fetuins by Glycopeptidase F Treatment. Glycopeptidase F treatment of bovine fetuin after tryptic digestion of the polypeptide chain results in almost complete release of N-glycosides (Townsend et al., 1989). Although the direct application of glycopeptidase F to a gly-

Table I: Carbohydrate Compositions of Fetuins from Different Species

	Fuc	Gal	Man[d]	GalNAc	GlcNAc
bovine fetuin[a]	–[c]	14.5	9	3.7	24.7
bovine fetuin[a]	–	14.9	9	3.9	24.1
bovine fetuin[b]	–	15.1	9	3.5	23.4
ovine fetuin	–	14.1	9	2.2	24.2
porcine fetuin	3.6	11.6	9	1.1	19.7
human α_2-HS	1.0	8.7	6	2.9	11.7

[a] Two preparations of bovine fetuin, each from a single calf (see Experimental Procedures). [b] A commercial preparation of bovine fetuin from GIBCO. [c] Not detected. [d] All values are normalized on the basis that bovine, ovine, and porcine fetuins have three complex type N-glycans (containing nine Man) per molecule and that human α_2-HS glycoprotein has two complex type N-glycans (containing six Man).

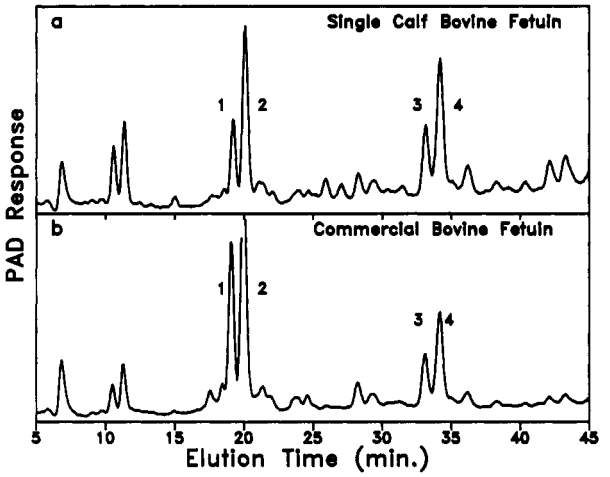


FIGURE 1: HPAEC of sialyloligosaccharides from bovine fetuins. Sialyloligosaccharides released by glycopeptidase F treatment of (a) a single-calf bovine fetuin and (b) a commercial preparation of bovine fetuin (GIBCO) were analyzed by HPAEC. Structural assignment of peaks 1, 2, 3, and 4 is shown in Figure 2. The chromatographic conditions are described in the text.

coprotein itself sometimes causes incomplete release of N-glycosides (Tarentino et al., 1985), the resulting oligosaccharide maps of fetuins (including human α_2-HS-glycoprotein) by the direct method were identical to those by the enzyme treatment after tryptic digestion.

Ratio of Gal4,4,4TRI and Gal4,4,3TRI in Two Single-Calf Preparations of Bovine Fetuin. The carbohydrate composition of two preparations of single-calf fetuin is shown in Table I. There is no large difference in carbohydrate composition among the two single-calf fetuins and a commercial fetuin. Figure 1 shows a comparison between the oligosaccharides released by glycopeptidase F digestion of one preparation of single-calf bovine fetuin and of a commercial fetuin (GIBCO). Another preparation of single-calf fetuin showed a chromatogram quite similar to Figure 1a. The major peaks in the chromatograms are identified by comparison with the reference oligosaccharides, and their structures are shown in Figure 2. The Gal4,4,3TRI structure in bovine fetuin is known to be mostly tetrasialylated while Gal4,4,4TRI tends to be trisialylated (Townsend et al., 1989). In Figure 1, the combined areas of peaks 1 and 2 (see Figure 2 for structures) approximately represent the total population of Gal4,4,4TRI structure and the combined areas of peaks 3 and 4 (see Figure 2 for structures) represent the total population of Gal4,4,3TRI. The comparison of parts a and b in Figure 1 shows that there is only a quantitative difference in the ratios of these structures.

Carbohydrate Composition of Fetuins from Different Species. Table I also compares the carbohydrate compositions of bovine, ovine, and porcine fetuins and human α_2-HS-glycoprotein. The composition is expressed in a normalized ratio on the basis of the presence of three complex type N-glycoside chains per molecule of the glycoprotein, thus nine residues of Man for fetuins and six residues of Man for human α_2-HS-glycoprotein, which has only two N-glycosides. Table I shows the close similarity of bovine and ovine fetuins. It also clearly shows the lower values of Gal and GlcNAc for porcine fetuin and human α_2-HS-glycoprotein, suggesting that they may have carbohydrate chains different from those of bovine and ovine fetuins. Porcine fetuin and α_2-HS-glycoprotein also differ in the Fuc value.

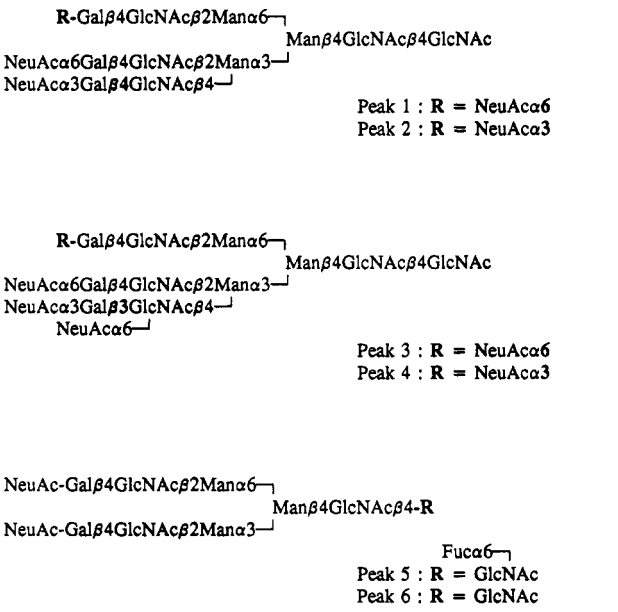


FIGURE 2: Structures of sialyloligosaccharides which appear as peaks 1–4 in Figure 1 and peaks 1–6 in Figure 3.

HPAEC Analysis of Oligosaccharides of Fetuins. Figure 3 shows the HPAE chromatograms of N-glycosides released by direct treatment of fetuins from different species with glycopeptidase F ("sialyloligosaccharide maps"). Apparently, the composition of N-glycans in ovine fetuin is quite similar to that in bovine fetuin. However, there are differences in the sialylation patterns of Gal4,4,4TRI and of Gal4,4,3TRI between N-glycosides of bovine and ovine fetuins (Figure 3a,b). NeuAcα3 linkage in the Galβ4GlcNAcβ2Manα6 branch exists in greater quantities than NeuAcα6 linkage for both Gal4,4,4TRI and Gal4,4,3TRI oligosaccharides in bovine fetuin, whereas the converse is the case for ovine fetuin. On the other hand, porcine fetuin and human α_2-HS-glycoprotein have N-glycoside chains of structures different from those of bovine and ovine fetuins (Figure 3c,d). The major peaks from these glycoproteins (peak 5 in Figure 3c and peak 6 in Figure 3d) were judged to be disialyloligosaccharides on the basis of their elution positions. The major component of porcine fetuin N-glycosides (peak 5) was identified as disialylated fucosyl biantennary complex type structure (Figure 2) and the major N-glycoside of human α_2-HS-glycoprotein (peak 6) was identified as disialyl biantennary structure (Figure 2) from the analysis of asialooligosaccharides (shown below).

After desialylation of these oligosaccharides, HPAEC analyses were performed to obtain asialooligosaccharide maps of these fetuins (Figure 4). The similarities and differences among the structures of N-glycans of fetuins from different species are more clearly shown here than in the sialyloligosaccharide maps (Figure 3). The major asialooligosaccharides of bovine and ovine fetuins, peaks A and B in Figure 4a,b, were identified as structures A and B shown in the abstract by

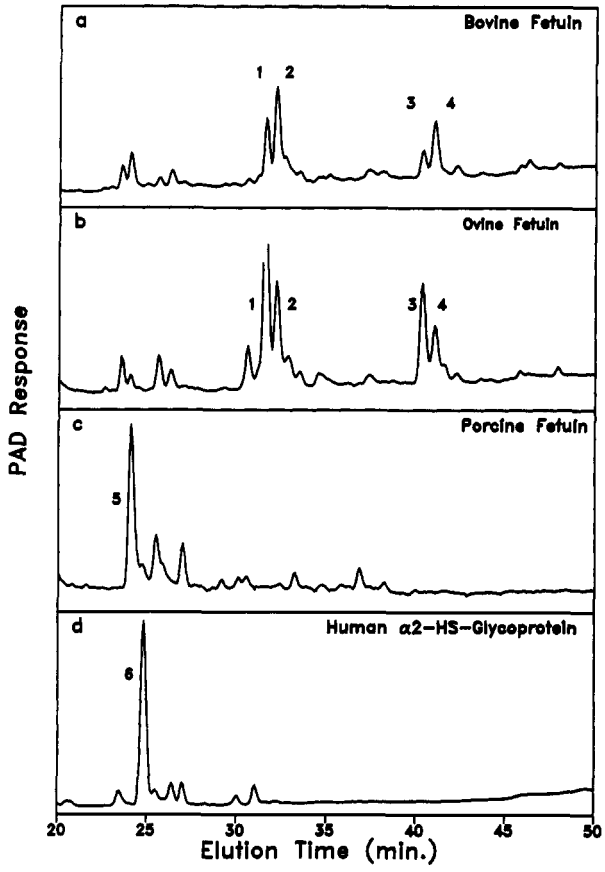


FIGURE 3: HPAEC of sialyloligosaccharides of fetuins from different species (sialyloligosaccharide maps). Sialyloligosaccharides of (a) bovine fetuin, (b) ovine fetuin, (c) porcine fetuin, and (d) human α_2-HS-glycoprotein were analyzed by HPAEC. Structural assignment of peaks 1–6 in the chromatograms is shown in Figure 2. The chromatographic conditions are described in the text.

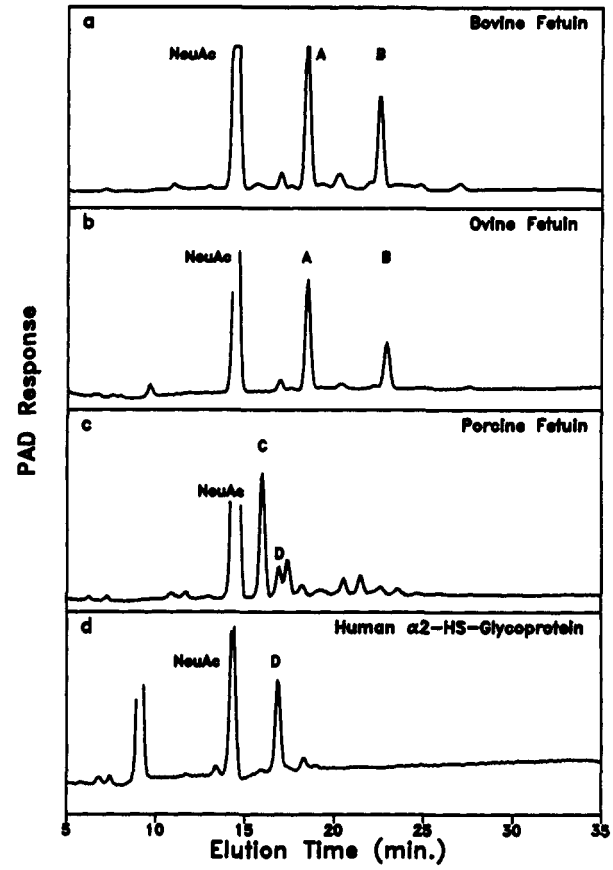


FIGURE 4: HPAEC of asialooligosaccharides of fetuins from different species (asialooligosaccharide maps). Desialylated oligosaccharides of (a) bovine fetuin, (b) ovine fetuin, (c) porcine fetuin, and (d) human α_2-HS-glycoprotein were analyzed by HPAEC. Structural assignment of peaks A–D in the chromatograms is shown in the abstract. The chromatographic conditions are described in the text. A large peak around 9 min in (d) is derived from residual 2-mercaptoethanol.

co-chromatography with the standard oligosaccharides. Again, bovine and ovine fetuins have very similar asialooligosaccharides, only slightly differing in the ratios of Gal4,4,4TRI (peak A) and Gal4,4,3TRI (peak B). Porcine fetuin and human α_2-HS-glycoprotein have oligosaccharides of structures quite different (Figure 4c,d) from those of bovine and ovine fetuins. The major component of asialooligosaccharides of human α_2-HS-glycoprotein (peak D in Figure 4d) was identified to be a biantennary complex type (structure D in the abstract) by its coelution with the standard oligosaccharide. The major peak in the asialooligosaccharide map of porcine fetuin (peak C in Figure 4c) co-chromatographed with the biantennary complex type oligosaccharide (peak D) after α-fucosidase treatment. This result suggests that porcine fetuin has a fucosylated biantennary structure as the major N-glycoside (structure C in the abstract). Also, Figure 4c shows the presence of a small amount of nonfucosylated biantennary complex type structure in N-glycosides of porcine fetuin. Other minor peaks were not identified.

PA Analysis of N-Glycosides from Porcine Fetuin and Human α_2-HS-Glycoprotein. To confirm the results shown above, pyridylamino (PA) derivatives of the asialooligosaccharides from porcine fetuin and human α_2-HS-glycoprotein were prepared and analyzed by reverse-phase HPLC and amide–silica gel HPLC. Figure 5 shows the result of the reverse-phase HPLC analysis. Two major peaks (E and F) in the chromatograms in Figure 5 were identified by co-chromatography with the standard PA-oligosaccharides. The major component of PA-oligosaccharides from porcine fetuin (peak F in Figure 5a) is the PA derivative of fucosyl biantennary complex type oligosaccharide (Figure 6). As was the case for nonderivatized asialooligosaccharides, α-fucosidase treatment of porcine fetuin PA-oligosaccharides resulted in a shift of peak F to the same elution position as that of the reference nonfucosylated biantennary compound (Figure 5b). Figure 5a also shows that porcine fetuin contains a small amount of nonfucosylated biantennary complex type structure (peak E), which is the major component of the N-glycosides from human α_2-HS-glycoprotein (Figure 5c). Analysis of these PA-oligosaccharides on amide–silica gel HPLC by co-chromatography with reference compounds also confirmed the correctness of the assignment (data not shown).

Fast Atom Bombardment Mass Spectrometry (FAB-MS). In order to confirm the identified structure of the major N-glycoside of porcine fetuin, FAB-MS of the PA-oligosaccharide was measured. Positive ions corresponding to $[M + H]^+$ (m/z = 1865.65) and $[M + Na]^+$ (m/z = 1887.62) were observed. This result agrees well with the calculated monoisotopic mass of this PA derivative (the structure of peak F in Figure 6), which is 1864.708.

Discussion

Bovine Fetuin from a Single Calf. We and others (Townsend et al., 1986; Takasaki & Kobata, 1986) have previously demonstrated the presence of two kinds of triantennary oligosaccharides, Gal4,4,4TRI and Gal4,4,3TRI (structures A and B in the abstract), in bovine fetuin. Subsequently, these two triantennary oligosaccharides in different fetuin preparations from different commercial sources were

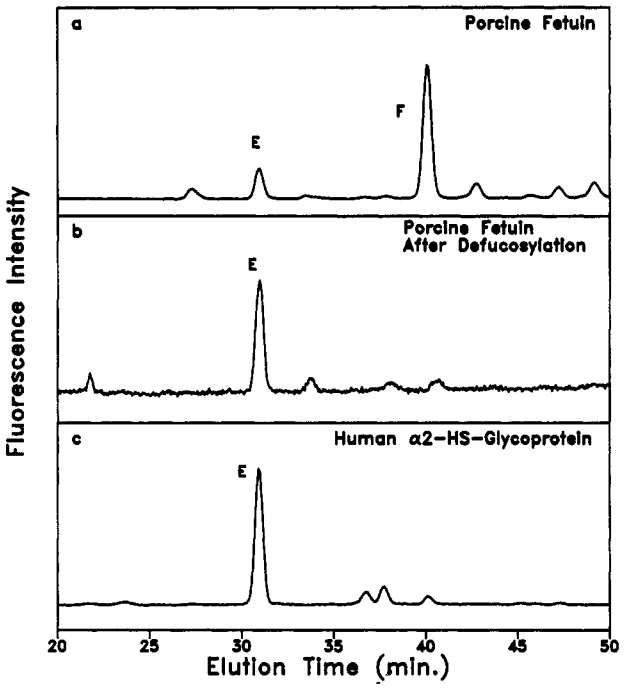
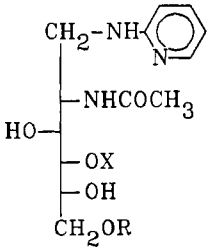


FIGURE 5: Reverse-phase HPLC of PA-oligosaccharides of fetuins from different species. Reverse-phase HPLC of (a) PA-oligosaccharides of porcine fetuin, (b) α-fucosidase digest of (a), and (c) PA-oligosaccharides of human α_2-HS-glycoprotein are shown. Structural assignment of peaks E and F is shown in Figure 6. The chromatographic conditions are described in the text.



R = H (peak E), Fucα6 (peak F)

X = Galβ4GlcNAcβ2Manα6
Manβ4GlcNAcβ4
Galβ4GlcNAcβ2Manα3

FIGURE 6: Structures of PA-oligosaccharides which appear as peaks E and F in Figure 5.

found to exist in different ratios (Townsend et al., 1989). It is fair to assume that the formation of Galβ3GlnNAc requires a different galactosyltransferase from the transferase that forms Galβ4GlcNAc. Thus, the ratio of Gal4,4,4TRI and Gal4,4,3TRI in fetuin must be under genetic control, and individual calves may express one or the other of these oligosaccharides.

When we examined two preparations of bovine fetuin isolated from two different single newborn calves, we found that the ratios of Gal4,4,4TRI and Gal4,4,3TRI in the two preparations are quite similar and also quite similar to that of one preparation of the commercial fetuin (GIBCO) prepared by the same procedure. Thus, it appears that in these calves both galactosyltransferases required to synthesize the two different structures coexist.

In should be noted that the different commercial fetuin preparations used in the earlier study (Townsend et al., 1989) were from different sources, and it is possible that the ratios of the Gal4,4,4TRI and Gal4,4,3TRI may be affected during the isolation procedure. Thus, it was necessary to ascertain that the ratio of the two structures could not change in the purification procedure. For this purpose, two additional preparations of bovine fetuin were prepared; one preparation was purified from pooled newborn calf serum (from GIBCO) by the same method as the two single calf fetuins, and the other was GIBCO fetuin further subjected to the same isolation procedure. The HPAEC of the oligosaccharides released from both preparations above were quite similar to those in Figure 1 (data not shown), suggesting that the ratio of Gal4,4,4TRI and Gal4,4,3TRI is preserved during the purification procedure.

Fetuins from Different Species. A close homology in amino acid sequences exists between bovine fetuin and human α_2-HS-glycoprotein in spite of the posttranslational cleavage in the C-terminal domain of human α_2-HS-glycoprotein (Figure 7), and thus α_2-HS-glycoprotein is considered to be a human counterpart of fetuin (Christie et al., 1987; Dziegielewska et al., 1990). Recently, cDNA sequences of ovine and porcine fetuins were elucidated, and they are also quite homologous to that of bovine fetuin (Figure 7; Brown et al., 1992). About 60% of the amino acids are conserved in the polypeptides of these four fetuins (including human α_2-HS-glycoprotein) and, especially, bovine and ovine fetuins have 94% homology. It is noteworthy that 12 cysteine residues in the four fetuins are located at identical positions and amino acid sequences around these cysteines are well conserved (Figure 7). This suggests that the gross tertiary structures of these fetuins are likely to be quite similar. Moreover, the positions of three N-glycosylation consensus sequences in the polypeptides are identical except for human α_2-HS-glycoprotein in which the first site (Asn 81) is replaced by Arg, and thus eliminating the possibility of N-glycosylation (Figure 7). Therefore, it is interesting to investigate structures of the oligosaccharides linked to Asn in the well-conserved sequences in these homologous glycoproteins from different origins.

Bovine fetuin oligosaccharides have been studied by a number of laboratories (Nilsson et al., 1979; Townsend et al., 1986, 1989; Takasaki & Kobata, 1986; Green et al., 1988), and their structural assignments have been extensively documented. We and others (Townsend et al., 1989; Lee, 1990; Spellman, 1991) have reported that sialyloligosaccharide maps and asialooligosaccharide maps by HPAEC (such as the chromatograms in Figures 3 and 4) are extremely valuable ways of structural analysis of glycoprotein-derived oligosaccharides because of their high resolution. The assignment of the major peaks in the HPAEC oligosaccharide maps of bovine and ovine fetuins (Figures 3 and 4) was established by co-chromatography with the reference oligosaccharides. In addition to the HPAEC analysis, we carried out HPLC analysis of PA derivatives (Tomiya et al., 1988) of the asialooligosaccharides from porcine fetuin and human α_2-HS-glycoprotein to further consolidate evidences for the identification of these oligosaccharides. Furthermore, the molecular weight of PA derivative of the major asialooligosaccharide from porcine fetuin measured by FAB-MS agreed with the identified structure.

We found that ovine fetuin has both Gal4,4,4TRI and Gal4,4,3TRI structures in a slightly different ratio from that of bovine fetuin and that theses triantennary complex type structures are the major N-glycosides. There is a difference in the sialylation pattern of those triantennary oligosaccharides between the fetuins from these species. To our surprise, N-glycosides of porcine fetuin and human α_2-HS-glycoprotein were drastically different from others. The predominant

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          1                                                  50
Bovine    IPLDPVAGYK EPACDDPDTE QAALAAVDYI NKHLPRGYKH TLNQIDSVKV
Ovine     IPLDPIAGYK EPACDDPDTE QAALAAVDYI NKHLPRGYKH TLNQIDSVKV
Porcine   VPHGPILGYR EPACDDVETE QAALAAVDYI NKHLPRGYKH TLNQVDSVKV
Human     APHGPGLIYR QPNCDDPETE EAALVAIDYI NQNLPWGYKH TLNQIDEVKV

                                          *                  100
Bovine    WPRRPTGEVY DIEIDTLETT CHVLDPTPLA NCSVRQQTQH AVEGDCDIHV
Ovine     WPRRPTGEVY DIEIDTLETT CHVLDPTPLV NCSVRQQTEH AVEGDCDIHV
Porcine   WPRRPAGEVF DIEIDTLETT CHVLDPTPLA NCSVRQLTEH AVEGDCDFHV
Human     WPQQPSGELF EIEIDTLETT CHVLDPTPVA RCSVRQLKEH AVEGDCDFQL

                                              *              150
Bovine    LKQDGQFSVL FTKCDSSPDS AEDVRKLCPD CPLLAPLNDS RVVHAVEVAL
Ovine     LKQDGQFSVL FTKCDSSPDS AEDVRKLCPD CPLLAPLNNS QVVHAAEVAL
Porcine   LKQDGQFSVL FAKCDSSPDS AEDVHKVCPN CPLLAPLNDS RVVHAAESAL
Human     LKLDGKFSVV YAKCDSSPDS AEDVRKVCQD CPLLAPLNDT RVVHAAKAAL

             *                                                 200
Bovine    ATFNAESNGS YLQLVEISRA QFVPLPVSVS VEFAVAATDC IAKEVVDPTK
Ovine     ATFNAQNNGS YFQLVEISRA QFVPLPGSVS VEFAVAATDC IAKEVVDPTK
Porcine   AAFNAQSNGS YLQLVEISRA QLVPLSASVS VEFAVAVTDC VAKEAYSPTK
Human     AAFNAQNNGS NFQLEEISRA QLVPLPPSTY VEFTVSGTDC VAKEATEAAK

                                                             250
Bovine    CNLLAEKQYG FCKGSVIQKA LGGEDVRVTC TLFQTQPVIP QPQPDGA-EA
Ovine     CNLLAEKQYG FCKGSVIQKA LGGEDVTVTC TLFQTQPVIP QPQPEGA-EA
Porcine   CNLLVEKQYG FCKGTVTAK- VNEEDVAVTC TVFQTQPVVL QPQPAGA-DA
Human     CNLLAEKQYG FCKATLSEK- LGGAEVAVTC TVFQTQPVTS QPQPEGANEA

                                                             300
Bovine    EAPSAVPDAA GP-----TPS AAGPPVAS-V VVGPSVVAVP LP-LHRAHYD
Ovine     GAPSAVPDAA VPDAAVPAPS AAGLPVGS-V VAGPSVVAVP LP-LHRAHYD
Porcine   GA-TPVVDAA ATASPLADVP AASLVVGPMV VAV----PPG IPPVHRSHYD
Human     V-PTPVVDPD APPSPPLGAP GLPPAGSPPD SHVL

                                                             350
Bovine    LRHTFSGVAS VESSSGEAFH VGKTPIVGQP SIPGG----P V-RLCPGRIR
Ovine     LRHTFSGVAS VESASGEAFH VGKTPIVGQP SVPGG----P V-HLCPGRIR
Porcine   LRHSFSGVAS VESASGEAFH VGKTPKGAQP SIPAADGSVP VVRPCPGRIR
Human                               TVVQP SVGAA--AGP VVPPCPGRIR


Bovine    YFKI
Ovine     YFKI
Porcine   HFKI
Human     HFKV
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FIGURE 7: Comparison of amino acid sequences of fetuins from different species (Dziegielewska et al., 1990; Brown et al., 1992; Lee et al., 1987). Amino acid sequences of bovine, ovine, and porcine fetuins and human α_2-HS-glycoprotein are shown. Gaps are introduced for improved alignment between four sequences. α_2-HS-glycoprotein appears in two-chain form, A-chain (282 residues) and B-chain (27 residues), due to a posttranslational cleavage of the pro-sequence (Lee et al., 1987). Sequences are expressed in the single-letter code. Asterisks indicate the N-glycosylation sites.

N-glycoside component in porcine fetuin was a biantennary complex type structure, fucosylated at the reducing terminal GlcNAc (Figure 2, the structure of peak 5), but there also exists a non-fucosylated biantennary complex type structure. Human α_2-HS-glycoprotein bears the N-glycoside of biantennary complex type structure almost exclusively. Our finding agrees with the concept of species specificity of N-glycan structure in glycoproteins from different species (Kobata, 1984).

It is known that the amino acid sequence of a glycoprotein can play an important role in determining its glycan type. N-Glycoside structures of several recombinant glycoproteins expressed in different cell lines showed that the type of N-glycan structure observed in a specific glycoprotein or at its specific glycosylation site is preserved in various host cells (Cumming, 1991, and references cited therein). For example, recombinant erythropoietin (EPO) produced by CHO cells or BHK cells and human urinary EPO have mainly tetraantennary complex type N-glycan, regardless of the cell type of expression (Sasaki et al., 1987; Takeuchi et al., 1988; Tsuda et al., 1988). On the other hand, when CHO cells were used as expression host cells of other recombinant glycoproteins such as tissue-type plasminogen activator (tPA) and interferon-β (IFN-β), they produced N-glycosides other than tetraantennary complex type (Spellman et al., 1989; Kagawa et al., 1988). The ratios of Gal4,4,4TRI and Gal4,4,3TRI at different N-glycosylation sites in bovine fetuin were quite different (Townsend et al., 1986; Rice et al., 1990), suggesting that the peptide sequence or conformational structure around the N-glycosylation site influences the glycosylation pattern.

In the case of the glycosylation of fetuins, it is stil not clear whether polypeptide sequence or host cell type plays the more

important role in determination of the branching pattern of glycans at specific glycosylation sites. For example, bovine fibrinogen has mainly biantennary complex type N-glycosides, although the triantennary structures are predominant in bovine fetuin N-glycosides. The absence of the third Galβ4/3GlcNAc branch in fibrinogen may mean that *N*-acetylglucosaminyltransferase IV (Gn-T IV) required for the formation of Gal4,4,4TRI and Gal4,4,3TRI (Gleeson & Schachter, 1983) is either absent or suppressed. Human plasma α_1-acid glycoprotein has mainly tri- and tetraantennary complex type N-glycosides (Yoshima et al., 1981), while human α_2-HS-glycoprotein (human fetuin) and human fibrinogen (Townsend et al., 1982) have mainly biantennary complex type N-glycosides. Also, human plasma α_1-protease inhibitor has both bi- and triantennary complex type N-glycosides (Mega et al., 1980). Again, it seems that activity of Gn-T IV or V is expressed in a different manner according to each glycoprotein. Since these four plasma glycoproteins are thought to be synthesized in liver, there should be some information in the peptide sequence of a glycoprotein, other than cell specificity, which determines its glycan branching pattern. At the present, the factor in a polypeptide chain which regulates the activity of Gn-T IV or V is not clear. However, one possibility is that these transferases could recognize a slight difference in the tertiary structure around an N-glycosylation site in polypeptide chain.

Thus, the species-specific N-glycan pattern of a glycoprotein is in part due to the species-specific expression of some glycosyltransferase(s), as well as to the difference in peptide sequence which affects the activity of the enzyme(s). In the case of N-glycosides of fetuins studied here, the core fucosylation of porcine fetuin N-glycoside is attributed to α1,6-fucosyltransferase activity in pig liver (Longmore & Schachter, 1982). However, the presence of the third Galβ4/3GlcNAc branch in the N-glycosides of bovine and ovine fetuins and its absence in porcine fetuin and human α_2-HS-glycoprotein may be due to the slight differencs in the peptide sequence and in the local conformation around the N-glycosylation sites, which Gn-T IV recognizes. In fact, there are three positions in the peptide sequence around N-glycosylation sites where porcine fetuin and human α_2-HS-glycoprotein are consistently different from bovine and ovine fetuins. They are residues 87 (L for Q), 148 (S/A for V), and 172 (L for F). Whether these amino acid changes can bring about the presence or absence of the third Galβ4/3GlcNAc branch, perhaps by influencing the activity of Gn-T IV, is not clear at the present.

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References

Brown, W. M., Dziegielewska, K. M., Saunders, N. R., Christie, D. L., Nawratil, P., & Müller-Esterl, W. (1992) *Eur. J. Biochem. 205*, 321–331.

Christie, D. L., Dziegielewska, K. M., Hill, R. M., & Saunders, N. R. (1987) *FEBS Lett. 214*, 45–49.

Cumming, D. A. (1991) *Glycobiology 1*, 115–130.

Dziegielewska, K. M., Evans, C. A. N., Fossan, G., Lorscheider, F. L., Malinowska, D. H., Møllgård, K., Reynolds, M. L., Saunders, N. R., & Wilkinson, S. (1980a) *J. Physiol. 300*, 441–455.

Dziegielewska, K. M., Kocsis, G., & Saunders, N. R. (1980b) *Comp. Biochem. Physiol. 66B*, 535–541.

Dziegielewska, K. M., Brown, W. M., Casey, S.-J., Christie, D. L., Foreman, R. C., Hill, R. M., & Saunders, N. R. (1990) *J. Biol. Chem. 265*, 4354–4357.

Gleeson, P. A., & Schachter, H. (1983) *J. Biol. Chem. 258*, 6162–6173.

Green, E. D., Adelt, G., Baenziger, J. U., Wilson, S., & van Halbeek, H. (1988) *J. Biol. Chem. 263*, 18253–18268.

Hardy, M. R., Townsend, R. R., & Lee, Y. C. (1988) *Anal. Biochem. 170*, 54–62.

Hase, S., Hara, S., & Matsushima, Y. (1979) *J. Biochem. (Tokyo) 85*, 217–220.

Kagawa, Y., Takasaki, S., Utsumi, J., Hosoi, K., Shimizu, H., Kochibe, N., & Kobata, A. (1988) *J. Biol. Chem. 263*, 17508–17515.

Kellermann, J., Haupt, H., Auerswald, E.-A., & Müller-Esterl, W. (1989) *J. Biol. Chem. 264*, 14121–14128.

Kobata, A. (1984) in *Biology of Carbohydrate* (Ginsburg, V., & Robbins, P. W., Eds.) Vol. 2, pp 87–161, John Wiley, New York.

Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., & Ikenaka, T. (1990) *Agric. Biol. Chem. 54*, 2169–2170.

Lee, C.-C., Bowman, B. H., & Yang, F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4403–4407.

Lee, Y. C. (1990) *Anal. Biochem. 189*, 151–162.

Longmore, G. D., & Schachter, H. (1982) *Carbohydr. Res. 100*, 365–392.

Marti, J., Aliau, S., Bonfils, C., Vigne, C., & Moretti, J. (1973) *Biochim. Biophys. Acta. 303*, 348–359.

McKelvy, J. F., & Lee, Y. C. (1969) *Arch. Biochem. Biophys. 132*, 99–110.

Mega, T., Lujan, E., & Yoshida, A. (1980) *J. Biol. Chem. 255*, 4057–4061.

Nilsson, B., Nordén, N. E., & Svensson, S. (1979) *J. Biol. Chem. 254*, 4545–4553.

Rice, K. G., Rao, N. B. N., & Lee, Y. C. (1990) *Anal. Biochem. 184*, 249–258.

Sasaki, H., Bothner, B., Dell, A., & Fukuda, M. (1987) *J. Biol. Chem. 262*, 12059–12076.

Spellman, M. W. (1990) *Anal. Chem. 62*, 1714–1722.

Spellman, M. W., Basa, L. J., Leonard, C. K., Chakel, J. A., O'Connor, J. V., Wilson, S., & van Halbeek, H. (1989) *J. Biol. Chem. 264*, 14100–14111.

Spiro, R. G. (1960) *J. Biol. Chem. 235*, 2860–2869.

Takasaki, S., & Kobata, A. (1986) *Biochemistry 25*, 5709–5715.

Takeuchi, M., Takasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N., & Kobata, A. (1988) *J. Biol. Chem. 263*, 3657–3663.

Tarentino, A. L., Gômez, C. M., & Plummer, T. H., Jr. (1985) *Biochemistry 24*, 4665–4671.

Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., & Takahashi, N. (1988) *Anal. Biochem. 171*, 73–90.

Townsend, R. R., Hilliker, E., Li, Y.-T., Laine, R. A., Bell, W. R., & Lee, Y. C. (1982) *J. Biol. Chem. 257*, 9704–9710.

Townsend, R. R., Hardy, M. R., Wong, T. C., & Lee, Y. C. (1986) *Biochemistry 25*, 5716–5725.

Townsend, R. R., Hardy, M. R., Cumming, D. A., Carver, J. P., & Bendiak, B. (1989) *Anal. Biochem. 182*, 1–8.

Tsuda, E., Goto, M., Murakami, A., Akai, K., Ueda, M., Kawanishi, G., Takahashi, N., Sasaki, R., Chiba, H., Ishihara, H., Mori, M., Tejima, S., Endo, S., & Arata, Y. (1988) *Biochemistry 27*, 5646–5654.

Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., & Kobata, A. (1981) *J. Biol. Chem. 256*, 8476–8484.