Presence of an O-Glycosidically Linked Hexasaccharide in Fetuin*

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Examination by gel filtration, thin layer and anion exchange chromatography of the O-linked carbohydrate units released from fetuin by alkaline borohydride treatment indicated the presence in this glycoprotein of an acidic glucosamine-containing hexasaccharide in addition to the previously described tetrasaccharides and trisaccharides.

The structure of the hexasaccharide was determined to be NeuAcα2-3Galβ1→3[NeuAcα2-3Galβ1→4GlcNAcβ1→6]GalNAc, on the basis of exoglycosidase digestion, periodate oxidation, and methylation analysis as well as hydrazine-nitrous acid fragmentation. The latter procedure when carried out on the reduced asialohexasaccharide yielded Gal-2-deoxygalactitol and Gal-anhydromannose which were shown to be derived, respectively, from Gal-β-N-acetylgalactosaminil and Gal-GlcNAc sequences. Reductive amination of the Gal-anhydromannose disaccharide with [14C]methylamine permitted identification of its linkage as 1→4. While Diplococcus pneumoniae endo-α-D-N-acetylglucosaminidase acting on asialofetuin released the sialic acid-free tetra- and trisaccharides (Galβ1→3Galβ1→4GlcNAcβ1→6)GalNAc, this enzyme did not cleave the peptide attachment of the asialohexasaccharide (Galβ1→3Galβ1→4GlcNAcβ1→6)GalNAc.

The number of O-linked hexa-, tetra-, and trisaccharides per fetuin molecule was determined to be 0.2, 0.7, and 2.1, respectively, on the basis of galactosaminil analyses. The absence of O-linked N-acetylglucosamine-containing tetra- or pentasaccharides in fetuin suggests that the attachment of this sugar is a rate-limiting step; furthermore, the limited occurrence of the hexasaccharide may indicate that the addition of sialic acid to Galβ1→3Galβ1→4GlcNAc to form the NeuAcα2-3Gal linkage precludes action of the GlcNAc transferase to form the branch point on the GalNAc residue.

Fetuin, one of the first glycoproteins to be isolated and characterized (1-4), has served as a model in numerous studies relating to the structure, biosynthesis, and function of glycoconjugates (5). Such investigations have been facilitated by the ready availability of this protein and the substantial information already obtained about its N- and O-glycosidically linked carbohydrate units (6-8).

The latter sugar chains which have been shown to include a trisaccharide (NeuAcα2-3Galβ1→3GalNAc) and a tetrasaccharide (NeuAcα2-3Galβ1→3[NeuAcα2-6]GalNAc) (6) have now been detected in a large number of cell-surface and soluble glycoproteins (9) and are biosynthetically related, with the trisaccharide serving as the precursor of the tetrasaccharide (10). While the asparagine-linked carbohydrate units of fetuin, which are predominantly of the complex triantennary type, have also been the subject of several investigations (2-4, 7, 8, 11) recent studies have revealed the occurrence of structural variants not previously recognized (12, 13).

The possibility that the O-linked saccharides of fetuin might likewise include other less abundant species was suggested by the observation that an unexpected N-acetylgalactosamine-containing saccharide fragment was formed by alkaline sulfite treatment of this glycoprotein (14). In the present report we provide evidence that such an additional O-linked carbohydrate unit does indeed occur in fetuin in the form of a hexasaccharide with a NeuAcα2-3Galβ1→3[NeuAcα2-3Galβ1→4GlcNAcβ1→6]GalNAc structure.

EXPERIMENTAL PROCEDURES

Preparation of Fetuin—Fetuin was isolated from pooled fetal calf serum by low temperature ethanol fractionation (1) or was purchased from GIBCO (Spiro method). Asialofetuin was prepared by mild acid hydrolysis as previously described (6).

Alkaline Borohydride Treatment of Fetuin and Isolation of Reduced Oligosaccharides—Fetuin (50 mg/ml) was incubated with 1 M NaBH₄ in 0.1 M NaOH for 72 h at 37 °C to prepare unlabeled oligosaccharides (6), while the radiolabeled components were obtained by treating 10 mg of fetuin in 100 μl of 0.1 M NaOH containing 1 M NaB[3H₄] (Du Pont New England Nuclear, adjusted to 81 mCi/mmol specific activity) under the same conditions.

After acidification with 1 N acetic acid, passage through Dowex 50-X2, 200-400 mesh (H⁺ form), and volatilization of the boric acid with methanol, the samples were applied to a column of Dowex 1-X2, 200-400 mesh (acetate form) from which the acidic oligosaccharides were eluted with 1 N formic acid while any neutral components were removed in the effluent and water wash.

The unlabeled acidic oligosaccharides were then fractionated on a column (1.8 x 130 cm) of Bio-Gel P-4 (400 mesh) equilibrated in 0.1 M pyridine acetate, pH 5.0, at a flow rate of 8 ml/h while 4-ml fractions were collected and analyzed for sialic acid.

Digestion with Glycosidases—Incubations of NaB[3H₄]-labeled oligosaccharides (5 x 10⁴ dpm) with various exoglycosidases were carried out in 100 μl of buffer at 37 °C for 48 h in the presence of toluene. Digestions with Clostridium perfringens neuraminidase (35 munits, Sigma, Type VIII), jack bean β-galactosidase (0.1 unit, Sigma, Type VII), and jack bean β-N-acetylgalactosaminidase (0.4 unit, Sigma) were performed in 0.1 M sodium acetate buffer, pH 5.0, while incubation with Escherichia coli β-galactosidase (1 unit, Sigma, Type VII) was...
carried out in 0.1 M sodium phosphate buffer, pH 7.0, containing 10 mM MgCl₂. The digestes were terminated by heating at 100 °C for 3 min and the samples were then desalted by passage through coupled columns of Dowex 50-X2 (200-400 mesh, H⁺ form) and Dowex 1-X2 (200-400 mesh, acetate form) prior to examination by thin layer chromatography. In the case of the neuraminidase-treated samples the Dowex 1 column was eluted with 1 N formic acid to recover any remaining acidic components and this fraction was combined prior to chromatography with the unabsorbed material from the Dowex 50 and Dowex 1 columns.

Neutral and acidic (0.5 mg) incubates were run on four millimeters of *Diplococcus pneumoniae* endo-α-N-acetylgalactosaminidase (Genzyme, O-glycanase) in 50 μl of 10 mM sodium citrate/phosphate buffer, pH 6.0, containing 1 mM calcium acetate and 10 mM galactono-1,4-lactone for 16 h at 37 °C. At the end of this period the samples and undigested controls were freeze-dried and deproteinized by extraction with 80% (v/v) ethanol. The extracts after evaporation of the solvent were then treated with 1 N solution of NaBH₄ (Du Pont-New England Nuclear, 8.1 Ci/mmol) in 100 μl of 0.2 M sodium borate buffer, pH 9.0, for 4 h at room temperature. The neutral sugars of the borohydride with acetic acid the samples were applied to a 1.0 × 0.5-cm charcoal-Celite (Daro G-60-Celite 635, 1.1 by weight) column (15) from which the enzyme-released neutral saccharides were eluted with 8 ml of 25% (v/v) ethanol after an 8-ml water wash.

Periodate Oxidation—The NaBH₄-reduced oligosaccharides (3 × 10⁵ dpm) were oxidized with sodium metaperiodate and then reduced with NaBH₄, as previously described (6). The acidic products were analyzed after 15 min (21) on a Hewlett-Packard Packard Model 390A computer integrator. The released aminoseptol, after elution from the Dowex 50 with HCl was reacylated with acetic anhydride (16) prior to chromatographic identification.

Threosaminol and Galβ1-3ThrNACh standards were prepared, respectively, by strong acid and mild acid hydrolysis of the NaBH₄-reduced periodate oxidation product of the 3H-labeled trisaccharide (N-acetylgalactosamine) unit (20) using [3H]methylation counting.

Methylation—Permethylation of unlabeled oligosaccharide (20 nmol) was accomplished by the procedure of Hakomori (17) and was followed by hydrolysis, reduction, and acetylation as described (18) except that the neutral sugar fraction was obtained by passing the hydrolysate through coupled columns of Dowex 50 (H⁺ form) and Dowex 1 (acetate form). The released aminoseptol, after elution from the Dowex 50 with HCl was reacylated with acetic anhydride (16) prior to chromatographic identification.

RESULTS

Oligosaccharides Released by Alkaline Borohydride Treatment of Fetuin—Examination by thin layer chromatography of the acidic saccharides released from two fetuin preparations by alkaline NaBH₄ treatment revealed three radiolabeled components (A, B, and C) which migrated in Solvent System A with an Rₑₙ₅₀₀ₐ₅₀₅ₐ₄₃ of 0.10, 0.20, and 0.78, respectively (Fig. 1). Components B and C represented the O-linked tetrasaccharide and trisaccharide units of fetuin (6), while component A appeared to be a previously unrecognized oligosaccharide. A similar oligosaccharide pattern was observed in the commercial fetuin preparation and in fetuin which had undergone additional purification by preparative polyacrylamide gel electrophoresis (data not shown).

Examination by thin layer chromatography of the neutral fraction obtained after alkaline NaBH₄ treatment revealed no radiolabeled components even when severalfold larger amounts than required for visualization of component A (Fig. 1) were applied to the plate (not shown).

Analysis of the acidic oligosaccharides after preparative separation by thin layer chromatography indicated that component A like components B and C contained its radiolabel solely in galactosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol. The radioactive saccharides which remained at the origin (component N) are believed to be derived from the N-linked oligosaccharides of fetuin as glucosaminitol.
Chromatography was carried out visualized by fluorography. Components respectively, the O-linked tetrasaccharide and N are discussed in the text. The distinctive nature of component was also evident on Dowex 1 chromatography where it eluted during a pyridine formate gradient at a position intermediate between that of the trisaccharide (component C) and tetrasaccharide (component B) (Fig. 2).

Fractionation of the unlabeled acidic oligosaccharides released from fetuin by alkaline NaB[3H]4 treatment. When this tetrasaccharide product of neuraminidase treatment the radiolabeled hexasaccharide was converted to a neutral component ($R_{max}$ = 0.49) which was distinct from the Galβ1-3GalNAcH$_2$ product ($R_{max}$ = 1.60) derived from sialic acid removal of the fetuin tetra- and trisaccharides (6) (Fig. 4). Limited neuraminidase digestion (30 min) of the hexasaccharide produced an acidic radiolabeled species ($R_{max}$ = 0.22, data not shown) which appeared to be a pentasaccharide intermediate in a stepwise removal of the 2 sialic acid residues.

When this tetrasaccharide product of neuraminidase treatment was digested with jack bean β-galactosidase a new component was produced with a chromatographic migration ($R_{max}$ = 0.90) consistent with a trisaccharide (Fig. 5, lane 3); removal of galactose from the hexasaccharide only took place.
The purified saccharides \((3 \times 10^4\text{ dpm})\) were subjected to thin layer chromatography for 10 h on a cellulose-coated plate in Solvent System A with or without prior neuraminidase (NANase) treatment. The digestion of hexasaccharide (HEXA), tetrasaccharide (TETRA), and trisaccharide (TRI), representing, respectively, components A, B, and C (Fig. 1) were carried out as described under "Experimental Procedures." The radiolabeled components were detected by fluorography and compared to the migration of \(\text{NaB}[\text{H}]_4\) reduced Gal\(\beta\)-1-3GalNAc (Gal-GalN) and lactose (arrow) standards.

if preceded by incubation with neuraminidase. Digestion of the galactosidase product with jack bean \(\beta\)-N-acetylgalactosaminidase yielded a disaccharide which comigrated with Gal\(\beta\)1-3GalNAcH\(_2\) (Fig. 5, lane 4); this component was found to be resistant to the action of the \(\beta\)-galactosidase from jack bean as well as \(E.\, coli\).

**Fig. 5.** Effect of sequential exoglycosidase digestions on the thin layer chromatographic migration of the \(\text{NaB}[\text{H}]_4\)-reduced hexasaccharide from fetuin. Chromatography was carried out on the untreated hexasaccharide (native) or after sequential digestion with neuraminidase (NANase), \(\beta\)-galactosidase (Galase), and \(\beta\)-N-acetylgalactosaminidase (GNase) as indicated by the designation above each lane and described under "Experimental Procedures." In each case, \(3 \times 10^4\text{ dpm}\) was applied to a cellulose-coated plate and chromatography was then carried out for 10 h in Solvent System A. The radiolabeled components were detected by fluorography; the position of migrated lactitol (arrow) and Gal\(\beta\)1-3GalNAcH\(_2\) (Gal-GalN) are indicated.

**Fig. 4.** Comparison of the effect of neuraminidase digestion on the thin layer chromatographic migration of alkaline \(\text{NaB}[\text{H}]_4\)-released oligosaccharides from fetuin. The purified saccharides \((3 \times 10^4\text{ dpm})\) were subjected to thin layer chromatography for 10 h on a cellulose-coated plate in Solvent System A with or without prior neuraminidase (NANase) treatment. The digestion of hexasaccharide (HEXA), tetrasaccharide (TETRA), and trisaccharide (TRI), representing, respectively, components A, B, and C (Fig. 1) were carried out as described under "Experimental Procedures." The radiolabeled components were detected by fluorography and compared to the migration of \(\text{NaB}[\text{H}]_4\) reduced Gal\(\beta\)1-3GalNAc (Gal-GalN) and lactose (arrow) standards.
**Fig. 6.** Effect of periodate oxidation on the NaB\(^{[3H]}\)-reduced hexasaccharide. The reduced products of periodate-treated hexasaccharide (3 × 10^5 dpm) with (+) or without (−) subsequent mild HCl hydrolysis were chromatographed on a cellulose-coated thin layer plate for 10 h in Solvent System A. The hydrolytic treatment converted an acidic product into a neutral component as described in text. The radiolabeled saccharides were visualized by fluorography; the positions to which standard Gal\(^{3}1-3\)GalNAc\(_{2}\) (arrow) and Gal\(^{3}1-3\)ThrNAc\(_{2}\) (Gal-Thr\(_{2}\)N) migrated are indicated.

**Fig. 7.** Thin layer chromatographic identification of NaB\(^{[3H]}\)-reduced products from hydrazine/nitrous acid-treated fetuin asialohexasaccharide (HEXA) and asialotetrasaccharide (TETRA). Chromatography was carried out on a cellulose-coated plate for 20 h in Solvent System B and the components were visualized by fluorography. Components Di-1 and Di-2 were eluted from preparative chromatograms for further study. The migration of standard lactitol is indicated (arrow); Gal\(^{3}1-4\)AnMan\(_{2}\) standard moved to the same position as Di-2.

Distinction between a C-3 and a C-4 substituted N-acetylglucosamine could not be made. The introduction of a \([\text{C}^{14}]\) methylamine group at C-1 by reductive amination of the nitrous acid product permitted its asymmetry to be preserved and made possible a chromatographic discrimination between the C-3- and C-4-substituted Gal\(^{\beta}1-3\)AnMan isomers (Fig. 9). The mobility of the 1-aminomethyl derivative of the disaccharide product from the hexasaccharide unit identified it as the 1-4 isomer (Fig. 9).

**Fig. 8.** Effect of β-galactosidase digestion on disaccharides produced by hydrazine/nitrous acid treatment of asialohexasaccharide. The chromatographically purified (Di-1 and Di-2) hydrazine/nitrous acid products (Fig. 7) were applied to a cellulose-coated plate with (+) or without (−) prior E. coli β-galactosidase treatment. Chromatography (2 × 10^4 dpm/lane) was carried out in Solvent System A for 3 h and the radiolabeled components were detected by fluorography. The migrations of AnMan\(_{2}\) (AnMan) and 2-deoxygalactitol (2-dGal) are indicated.

**Fig. 9.** Thin layer chromatographic distinction between the β1-3 and β1-4 isomers of Gal\(^{\beta}1-3\)AnMan after reductive amination with \([\text{C}^{14}]\)methylamine. The migration of the Gal\(^{\beta}1-3\)AnMan product from the hydrazine/nitrous acid-treated asialohexasaccharide (HEXA) is compared to that of Gal\(^{\beta}1-3\)AnMan and Gal\(^{\beta}1-4\)AnMan standards after reductive amination with \([\text{C}^{14}]\)methylamine as described under "Experimental Procedures." Chromatography was performed on a cellulose-coated plate in Solvent System A for 6 h and the radiolabeled components were detected by fluorography. The migration of the lactitol standard is indicated (arrow).
endoglycosidase, in contrast to the Galβ1-3GalNAc originating from the fetuin tetra- and trisaccharides, is consistent with the restricted specificity of this enzyme (29).

**DISCUSSION**

The widespread use of fetuin as a model glycoprotein in biological and biochemical investigations makes a thorough understanding of the structure of its N- and O-linked saccharide units a matter of considerable importance. The present study contributes to our knowledge of this protein by demonstrating the occurrence of a previously undetected O-glycosidically linked hexasaccharide. After release by alkaline borohydride treatment this distinctive carbohydrate unit could be resolved from the O-linked tetra- and trisaccharides (6) by Bio-Gel P-4 filtration as well as by thin layer chromatography.

Our investigations have shown that the hexasaccharide (Fig. 10) is structurally related to the fetuin trisaccharide (NeuAc2-3Galβ1-3GalNAcH2) and tetrasaccharide (NeuAc2-3Galβ1-3[NeuAc2-6]GalNAcH2) units, differing from the latter by the occurrence of a sialyl-N-acetyllactosamine branch on C-6 of the N-acetylgalactosaminitol instead of a sialic acid residue. Indeed the structure of the hexasaccharide indicates that the NeuAc2-3Galβ1-4GlcNAc sequence is not restricted to the N-linked carbohydrate units of fetuin (3, 4).

The structural formulation for the hexasaccharide is based on the results of periodate oxidation, methylation, and glycosidase digestions; furthermore, our studies were facilitated by the employment of hydrazine/nitric acid treatment to fragment the asialohexasaccharide into its constituent disaccharide units by deaminative cleavage of the bond between glucoamine and galactosaminitol (Fig. 10). Reductive amination with [14C]methylamine of the Gal-AnMan product released by this treatment made it possible to distinguish between its β1-3 and β1-4 isomers. The other disaccharide obtained from the hydrazine/nitric acid degradation, namely Gal1-3dGalH2 in which the N-acetylgalactosaminitol residue of the hexasaccharide had been converted to 2-deoxygalactitol, permitted us to evaluate the anomeric configuration of the Gal1-3GalNAc2H2 bond since it was readily cleaved by β-galactosidase in contrast to the known (30) glycosidase resistance of Gal1-3GalNAcH2.

Although the observation that the hexasaccharide was released along with the tetra- and trisaccharides by β-elimination indicates that it is attached to a serine or threonine residue, the resistance of the asialohexasaccharide to cleavage by endo-α-N-acetylgalactosaminidase, which is consistent with the restricted specificity of this enzyme (29), precluded an assignment of the anomeric configuration of its linkage to the peptide chain.

An accounting of the O-glycosidically linked units of fetuin indicates that the hexasaccharide, like the tetrasaccharide, is present in submolar amounts (Table I) and therefore would be present in only a minority of fetuin molecules. This tabulation moreover raises the possibility that the hexa- and tetrasaccharide could occupy the same glycosylation site; the latter oligosaccharide has been previously shown to be attached only to serine in contrast to the two trisaccharide units which are linked to a serine and threonine residue in close proximity on the peptide chain (6).

Since the hexasaccharide was found in all preparations of fetuin purified by the low temperature ethanol fractionation procedure as well as in a preparation eluted from polyacrylamide electrophoresis gel there is no reason to doubt that it is an integral saccharide unit of this glycoprotein. Indeed a comparable occurrence in fetuin of N-linked carbohydrate unit variants in substantially less than molar amounts has recently been reported (12, 13).

Carbohydrate units of the O-linked type which contain a GlcNAcβ1-6GalNAc branch point as in the fetuin hexasaccharide, have been observed in a variety of secreted and membrane glycoproteins (9) and indeed a glycosyltransferase which functions to attach N-acetylglucosamine to C-6 of O-glycosidically linked N-acetylgalactosamine has been found in canine submaxillary glands (31) as well as a number of other tissues (32). The absence in fetuin of O-linked pentasaccharides containing the incomplete sialyl-N-acetyllactosamine sequence, such as have been detected in other glycoproteins (33-35), suggests that the attachment of the N-acetylgalactosamine is the rate-limiting step in the assembly of this branch of the fetuin hexasaccharide unit. If the O-linked hexa- and tetrasaccharides do indeed occupy the same glycosylation site, the relatively small amount of the former might reflect a competition between the α2-6-sialyltransferase and the β1-6GlcNAc transferase for a common NeuAc2-3Galβ1-3GalNAcNac substrate. If, however, in fetal calf liver the β1-6GlcNAc transferase acts specifically on O-linked Galβ1-3GalNAc, as it does in other tissues (31, 32), the predominating activity of the α2-3-sialyltransferase in this organ (10) may make this disaccharide acceptor a very transitory intermediate. Indeed in this case the β1-6GlcNAc transferase may paradoxically be in competition with the α2-3 rather than the α2-6-sialyltransferase as the latter enzyme has been shown to act exclusively on the NeuAc2-3Galβ1-3GalNAcNac trisaccharide in the fetal liver (10). The possibility that the sequence of assembly of the fetuin tetrasaccharide and hexasaccharide units differ in these salient respects is currently being explored as it may account for the relative proportion of the three O-linked carbohydrate units in the fetuin molecule.

Aside from its relevance to an understanding of the biosynthesis of O-linked saccharide chains the identification and characterization of a hexasaccharide unit in fetuin may prove to be of importance in the continued utilization of this glycoprotein as a model in biological and biochemical investigations.

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**Table 1**

| Oligosaccharide | Number of units | Composition |
|-----------------|----------------|-------------|
| Trisaccharide   | 2.13           | NeuAc 2,3  |
|                 |               | Gal 1,4    |
| Tetrasaccharide | 0.72           | NeuAc 2,3  |
|                 |               | Gal 1,4    |
| Hexasaccharide  | 0.15           | NeuAc 2,3  |
|                 |               | Gal 1,4    |

*Expressed as mole/mol of oligosaccharide; values for the tri- and tetrasaccharides have been previously reported (6).
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