Structure of the Complex Oligosaccharides of Fetuin

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The complete structure of the complex oligosaccharides of fetuin has been established. The three fractions of complex oligosaccharide which were isolated by ion exchange chromatography following pronase digestion (F-I, F-II, and F-III) had identical molar ratios of sialic acid (Sia), galactose, mannose, and N-acetylglucosamine of 3:3:3:5. A combination of methylation analyses, Smith periodate degradations, and endoglycosidase and exoglycosidase digestions were utilized to establish the structure which is proposed to be:

\[
\begin{align*}
\text{Sia}^2 &\rightarrow 3 \text{Gal}^1 \rightarrow 4 \text{GlcNAc}^1 \\
\text{Gal}^1 &\rightarrow 4 \text{GlcNAc}^1 \rightarrow \text{Asn} \\
\text{Sia}^2 &\rightarrow 6 \text{Gal}^1 \rightarrow 4 \text{GlcNAc}^1 \\
\text{Gal}^1 &\rightarrow 4 \text{GlcNAc}^1 \rightarrow 2 \text{Man}^1 \\
\text{Sia}^2 &\rightarrow 3 \text{Gal}^1 \rightarrow 4 \text{GlcNAc}^1 \\
\text{Gal}^1 &\rightarrow 4 \text{GlcNAc}^1 ightarrow 2 \text{Man}^1
\end{align*}
\]

Features of this structure not previously established include the presence of 2 residues of \(\alpha2,3\)- and 1 residue of \(\alpha2,6\)-linked sialic acid and their location relative to the mannose branch points. Also unusual is the presence of an \(\alpha\)-linked branch mannose with substituents at positions 2 and 4 which is in turn linked to position 6 of the \(\beta\)-linked, branch mannose. These features result in unexpected resistance to specific exoglycosidases.

EXPERIMENTAL PROCEDURES

RESULTS

Composition, Molecular Weight, Methylation, and Periodate Oxidation of Fetuin Complex Glycopeptides—The carbohydrate compositions of F-I, F-II, and F-III obtained by setting mannose to 3.0 residues are given in Table II. As indicated earlier, F-I and F-II were identical in virtually all respects and will be treated as a single entity. In Table III, the calculated molecular weight obtained by setting mannose to 3.0 residues (2974) can be seen to agree well with the estimated molecular weight of 2925 obtained by gel filtration on a calibrated Bio-Gel P-4 column (Fig. 2). The methylation analysis in Table IV of the intact glycopeptide demonstrates that there are two branch points involving mannose residues resulting in 1 residue each of 3,6- and 2,4-dimethyl mannose and that 2 of the 3 galactose residues, which are penultimate to sialic acid residues, are substituted at position 3 while 1 is substituted at position 6. All 5 of the N-acetylgalactosamine residues are substituted at position 4. In agreement with the features of exoglycosidases commonly utilized for such structural studies.

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1 Portions of this paper (including “Experimental Procedures,” Figs. 1 to 3, 7, and 8, Tables I to V, and “References”) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, Request Document No. 78M1302, cite author(s), and include a check or money order for $2.40 per set of photocopies.
methylation data, periodate oxidation of the intact glycopeptide (Table II) resulted in destruction of only 1 of 3 residues of mannose and 1 of 3 residues of galactose.

**Sequential Enzymatic Degradation**—Sequential or combined digestion with neuraminidase (C.P.), β-galactosidase (D.P.), and β-N-acetylglucosaminidase (D.P.) resulted in the release of 3 residues of sialic acid but never more than 2 residues of N-acetylglucosamine and between 2 and 2.5 residues of galactose (Table V). Reisolated and analysis of the remaining glycopeptide fragment confirmed the continued presence of 0.5 to 1.0 residue of galactose and 3 residues of N-acetylglucosamine. Repeated treatment of the reisolated core digestion with neuraminidase (C.P.), β-galactosidase (D.P.), and β-N-acetylglucosaminidase (D.P.) did not release more galactose or N-acetylglucosamine. Extended treatment of the glycopeptide core with jack bean β-galactosidase and β-N-acetylglucosaminidase resulted in the release of additional galactose at a slow rate but no additional N-acetylglucosamine could be released (data not shown). α-Mannosidase (J.B.) released 1 residue of mannose at this stage (Table V). Attempts to release galactose, N-acetylglucosamine, and mannose in any but the order presented in Table V were unsuccessful.

In agreement with these results were the estimated molecular weights obtained for iodinated fetuin glycopeptide and its degradation products (Fig. 2 and Table III) by gel filtration on a calibrated column of Bio-Gel P-4. The estimated molecular weights in Table III agree well with the calculated values based on the enzyme release data in Table V. Had 3 galactose residues been released, a glycopeptide (GlcNAc₂-Man₃, GlcNAc₂-Asn) of Mᵣ = 1615 would have resulted, and removal of 2 N-acetylglucosamine residues would have produced a glycopeptide (GlcNAc₂-Man₃, GlcNAc₂-Asn) of Mᵣ = 1209. Both of these values are considerably less than the estimated molecular weights of 1900 and 1450 obtained for the products of β-galactosidase and β-N-acetylglucosaminidase digestion. The resistance of the 3rd galactose residue of the fetuin glycopeptide to release by β-galactosidase (D.P.) was virtually complete when digests of iodinated glycopeptide were performed in the range of 1 to 100 pmol in 50 μl.

In order to further characterize the product of enzymatic digestion with neuraminidase, β-galactosidase (D.P.), and β-N-acetylglucosaminidase (D.P.), the methylation analysis shown in Table IV was performed. The methylation pattern indicated that the mannose substituted at position 2 had become terminal in agreement with the finding that α-mannosidase (J.B.) could now release 1 residue of mannose (Table V). Attempts to release galactose, N-acetylglucosamine, and mannose in any but the order presented in Table V were unsuccessful.

As will become apparent from the following data, Structure A is in fact the correct one.

The assignment of the terminal, α-linked mannose as originating from position 3 rather than position 6 of the underlying mannose in Structure A is consistent with the finding by Tai et al. (25) that endo-β-N-acetylglucosaminidase (D.P.) has a strict specificity for glycopeptides with structure

\[ R₁ \rightarrow 3 \rightarrow R₂ \rightarrow 4 \rightarrow \text{GlcNAc} → \text{Asn} \]

where R₁ is Manα → or oligosaccharide → Manα →. We have examined a number of monosialylated complex oligosaccharides and reconfirmed our original observation (23, 24) that the sialic acid in these structures is present on the branch arising from position 3 of the core mannose. In such cases, we have found that even when the α₁,6-linked mannose is terminal, the oligosaccharide remains resistant to endo-β-N-acetylglucosaminidase (D.P.) digestion; thus glycopeptides with the sequence

\[ R₁ \rightarrow 3 \rightarrow \text{Manβ₁} \rightarrow 4 \rightarrow \text{GlcNAcβ₁} \rightarrow 4 \rightarrow \text{GlcNAc} → \text{Asn} \]

where R₁ is Manα → or oligosaccharide → Manα →. We have established the sequence and anomeric linkage of the sugars in F₁ and F₂. The reduced oligosaccharides were digested with a number of exoglycosidases and the products separated by descending paper chromatography as described.
in Fig. 4. Neither F₁ nor F₂ was sensitive to β-galactosidase (D.P.), β-N-acetylglucosaminidase (D.P.), α-galactosidase, or α-N-acetylglucosaminidase. Treatment of F₁ with jack bean β-galactosidase resulted in a peak which co-migrated with F₂, further confirming that F₁ differed only in the presence of a terminal β-linked galactose residue. Treatment of F₂ with α-mannosidase (J.B.) (Fig. 4, Panel d) resulted in the release of mannose as predicted from the α-mannosidase digestion of the glycopeptide (see Table V). Of note is the fact that the fetuin oligosaccharides F₁, F₂, and F₃ had a slightly greater mobility than the corresponding bovine IgG oligosaccharides G₁, G₂, and G₃. These oligosaccharides differ only in that the N-acetylglucosamine→mannose linkage is β1,4 in the fetuin oligosaccharides and β1,2 in the bovine IgG oligosaccharides. Treatment of F₁ with jack bean β-N-acetylglucosaminidase produced an oligosaccharide (F₃) which co-migrated with the oligosaccharide from bovine IgG (G₃) having the structure shown in Fig. 4, Panel c. Treatment of F₂ with α-mannosidase produced an oligosaccharide which co-migrated with authentic Man₆GlcNAc₂ and was susceptible to α-mannosidase to produce N-acetylglucosaminitol. In addition, the structures of F₁, F₂, F₃, and F₄ were confirmed by methylation analysis as shown in Table IV.

Therefore, the structure of the fetuin complex oligosaccharides exclusive of the sialic acid residues is shown in Fig. 5.

**Location of the Sialic Acid Residues**—In order to locate the sialic acid residues, the oligosaccharide was subjected to periodate oxidation, reduction, and mild acid hydrolysis (0.05 N H₂SO₄/80°C/1 h) followed by reisolation of the glycopeptide core. The composition of the reisolated material (Table V) was identical with that of the periodate oxidized glycopeptide (Table II) which had not been reisolated. This indicated that the mild acid hydrolysis had not released any sugar despite the destruction of 1 residue of 2-substituted mannose. Methylation of the reisolated product of the Smith degradation (Table IV) demonstrated that the 3,4,6-trimethyl-mannose had been completely destroyed; however, 1 residue each of 3,6- and 2,4-dimethyl mannose were still present. In addition, as would be predicted from the methylation of the intact glycopeptide and the structure shown in Table IV, 2 residues of terminal 2,3,4,6-tetramethyl galactose and 1 residue of terminal 3,4,6-trimethyl-N-acetylglucosamine were now present. The reason for the continued presence of the 2,4-dimethyl mannose which should have been converted to a 2,3,4-trimethyl mannose appears to be resistance of the oxidized and reduced mannose product to hydrolysis. Increasing the sulfuric acid concentration to 0.1 N was also not effective in releasing the destroyed mannose residue. Treatment of the Smith degradation product with β-N-acetylglucosaminidase (D.P.) released 1 residue of N-acetylglucosamine (Table V) and converted the 3,6-dimethyl mannose to a 2,3,6-trimethyl mannose, indicating that the sialic acid-linked α2,6 was located on the branch originating from position 2 of the α-linked branch mannose. Further treatment with β-galactosidase (D.P.) and β-N-acetylglucosaminidase (D.P.) (Table V) resulted in the release of 1.4 residues of galactose and 0.7 residue of N-acetylglucosamine. Methylation of this product (Table IV) revealed the presence of a full residue of terminal (3,4,6-trimethyl)-N-acetylglucosamine and a full residue of...
2,3,6-trimethyl mannose, confirming the resistance of the N-acetylglucosamine $\beta$1 to 4 mannose $\alpha$ linkage to $\beta$-N-acetylglucosaminidase (D.P.) in the Smith degradation product. Thus, the complete sequence of the complex fetuin glycopeptides can be surmised to be that shown in Fig. 6.

**DISCUSSION**

The proposed structure for the complex oligosaccharides of fetuin has a number of interesting features. This oligosaccharide is an example of a three-branch pattern with the branch points involving one $\alpha$-linked and one $\beta$-linked mannose. The branching pattern is quite specific in that the $\alpha$-linked mannose constituting the second branch point arises exclusively from position 6 of the $\beta$-linked mannose residue.

This branching pattern has also been reported by Kondo et al. (27) for the $\beta$-linked mannose of the complex unit B oligosaccharide of porcine thyroglobulin; however, in the thyroglobulin oligosaccharide the substituents of the $\alpha$-linked, branch mannose are located at positions 3 and 6, whereas in fetuin they are located at positions 2 and 4. Kornfeld (28) established the opposite branching pattern for a glycopeptide (B-III) isolated from calf thymus membranes, where the $\alpha$-linked, branch mannose-bearing substituents at positions 3 and 6 arises from position 3 of the $\beta$-linked, branch mannose. This glycopeptide has a fourth branch which arises from an N-acetylglucosamine with substituents at positions 3 and 4.

In contrast, Tai et al. (29) have characterized a “hybrid” type of oligosaccharide moiety (GPIII-C) present on ovalbumin (Fig. 7) which, like the fetuin complex, oligosaccharide, has an $\alpha$-linked mannose serving as a branch point with $\beta$-linked N-acetylglucosaminyl substituents at positions 2 and 4; however, in the case of ovalbumin GPIII-C, this $\alpha$-linked mannose arises from position 3 of the $\beta$-linked mannose rather than position 6 as is the case in the fetuin glycopeptide. Of interest in regard to the branching patterns of each completely oligosaccharides is the finding by Li et al. (30) that the oligosaccharide lipid intermediate which appears to be processed into either simple or complex oligosaccharides following transfer to the nascent peptide, also has an asymmetric branching pattern. The basis for the control of the branching patterns of complex and simple oligosaccharides by “processing” of the lipid oligosaccharide following transfer of the oligosaccharide to the peptide remains at present unknown but appears to be highly specific. It is also apparent from these examples that a larger number of structures are becoming established which differ primarily in their branching pattern and which may have unique properties because of such differences.

The location of the sialic acid residues is also of considerable interest. Fetuin is unusual because 2 of the 3 sialic acid residues are linked to position 3 of underlying glycolactose residues while 1 is linked to position 6 of galactose. The $\alpha$2,6-linked sialic acid is located on the branch which arises from position 2 of the $\alpha$-linked, branch mannose, while the $\alpha$2,3-linked sialic acid residues are located on the branches arising from position 4 of the branched, $\alpha$-linked mannose and position 2 of the unbranched, $\alpha$-linked mannose (Fig. 6). The unique relationship of each of the 3 sialic acid residues to the underlying structure suggests that as many as three neuraminyltransferases could be involved in the synthesis of this oligosaccharide. Evidence for a high degree of specificity for neuraminyltransferase activity with respect to the branching pattern of complex oligosaccharides was also noted for immunoglobulins. Monosialylated complex oligosaccharides from IgA1, IgE, bovine IgG, and J chain (23, 24) always bear the single sialic acid constituent on the branch arising from position 3 of the $\beta$-linked core mannose. Tai et al. (31) found the opposite orientation for the galactose in nonsialylated bovine IgG oligosaccharides which have only a single galactose residue; i.e., the terminal galactose residue was located on the branch arising from position 6 of the $\alpha$-linked mannose in the core. We have confirmed this for human IgG as well. Such structural information clearly indicates that a high degree of specificity is involved in the addition of terminal sugars by glycosyltransferases, a finding which is in keeping with recent evidence for recognition of terminal sugars by receptors in hepatocytes, macrophages, and fibroblasts (32-37).

A number of unexpected exoglycosidase specificities were encountered in this study. First was the resistance of the galactose on the branch with the sequence Gal1 → 4GlcNAcβ1 → 4Manα1 → to both diplococcal and jack bean $\beta$-galactosidase. The basis for this is unclear since, in the case of $\beta$-galactosidase (D.P.), as much as 0.5 residue of this galactose could be released; however, no subsequent release was obtained when either the glycopeptide or the endoglycosidase-produced oligosaccharide alcohol Fi was treated repeatedly with $\beta$-galactosidase (D.P.). The galactose residue was also relatively resistant to jack bean $\beta$-galactosidase while in the glycopeptide form (with extended digestions more galactose could be released at a slow rate). On the other hand, $\beta$-galactosidase (J.B.) rapidly released the galactose from the endoglycosidase product F. The pattern of release suggests that removal of one or both of the nonresistant galactose residues may result in complete resistance to removal by $\beta$-galactosidase (D.P.) of the galactose residue arising from position 4 of the $\alpha$-linked mannose, whereas this same galactose residue may be sensitive to the $\beta$-galactosidase (D.P.) if it is attacked before one or both of the other galactose residues have been removed. The greatly increased sensitivity of this galactose residue to $\beta$-galactosidase (J.B.) following endoglycosidase treatment indicates that some feature of the core sugars or peptide may also significantly influence the ability of $\beta$-galactosidase (J.B.) to release galactose from the Gal1 → 4GlcNAcβ1 → sequence.

The N-acetylglucosamine linked $\beta$1,4 to the underlying $\alpha$-linked, branch mannose (GlcNAcβ1 → 4Manα1 → 6Manβ1 →) was completely resistant to both diplococcal and jack bean $\beta$ N-acetylglucosaminidase while in the glycopeptide form. Following endoglycosidase treatment and reduction with sodium borohydride, the terminal N-acetylglucosamine of oligosaccharide F13 remained resistant to both diplococcal $\beta$-N-acetylglucosaminidase, while it was rapidly and completely released by jack bean $\beta$-N-acetylglucosaminidase. This pattern of resistance was confirmed by examining the ability of concanavalin A immobilized on Sepharose (ConA-Sepharose) to bind either the oligosaccharide or the glycopeptide. While the oligosaccharide F13 was not bound by ConA-Sepharose, the product of jack bean $\beta$-N-acetylglucosaminidase treatment, oligosaccharide F13, was completely bound. In contrast, the glycopeptide from which F13 was derived was not bound by ConA-Sepharose and could not be converted to a form which would bind by exhaustive treatment with either jack bean or diplococcal $\beta$-N-acetylglucosaminidase, indicating that the terminal $\beta$1,4-linked N-acetylglucosamine had not been removed.

The resistance of GlcNAcβ1 → 4Man → to release with diplococcal $\beta$-N-acetylglucosaminidase is in marked contrast to the results obtained with the IgA glycopeptide II-A, the structure of which is shown in Fig. 8. The terminal $\beta$-N-acetylglucosamine of II-A which is linked $\beta$1,4 to the $\beta$-linked, core mannose is resistant to both diplococcal and jack bean $\beta$-N-acetylglucosaminidase. Following removal of the terminal galactose residue, the diplococcal enzyme releases only the non-terminal $\beta$1,2-linked N-acetylglucosamine residue. In contrast, following removal of the sialic acid and both galactose resi-
dyes, the diplococcal β-N-acetylglucosaminidase is able to release both β1,2-linked N-acetylhexosamine residues and the β1,4-linked residue. Clearly, aspects of the oligosaccharide structure in addition to anomeric linkage and position of the substituents on the penultimate sugar can influence the ability of exoglycosidases to release a terminal sugar. In the case of the diplococcal β-N-acetylglucosaminidase, the anomeric configuration of the underlying mannose (α versus β) may be critical or some other aspect of the structure may be influential. Although such differences in specificity can be utilized to good advantage in structural studies as has been the case with endoglycosidases, the inability to release a suspected terminal sugar must be examined closely before concluding that it is nonterminal or is of a different anomeric linkage. This also important when attempting to degrade intact glycoproteins with exoglycosidases.

REFERENCES

References are found on p. 790.
### Table IV

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<th>Glycopeptide</th>
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### Table V

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### Figure 1

Isolation of N-glycosidically-linked complex oligosaccharides from fetuin. A. SD-LC/IRP chromatography of a 2.5 x 30 cm column of Bio-Gel P-2 (200-400 mesh) in 0.1 M ammonium bicarbonate. Equal amounts of each fraction were assayed for hexoses by the 3,5-dinitrosalicylic acid (DNS) assay and for sialic acid by the thiobarbituric acid assay (TBA). Pool A contained predominantly N-glycolyl galactosamines while pool B contained predominantly N-glycolyl glucosamines. B. Chromatography of pool A on Bio-Gel P-4 as above. C. Chromatography of pool B on Bio-Gel P-4 as above.
Figure 2. A plot of molecular weights of Bio-Gel P-4 (in base 1000) on the x-axis and distance from origin on the y-axis for the separation of fetuin oligosaccharides. The distance from origin and molecular weight are both on a logarithmic scale.

Figure 3. A diagram showing the separation of fetuin oligosaccharides on Bio-Gel P-4. The oligosaccharides are fractionated into different peaks based on their size and shape.

Figure 4. A simplified diagram of the proposed structure of fetuin oligosaccharide as established by Tei et al. (1980).
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