Structural Studies of the Endoglycosidase H-resistant Oligosaccharides Present on Human β-Glucuronidase*

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Human β-glucuronidase bears 3–4 oligosaccharide moieties/subunit of M = 75,000. We have previously characterized the endoglycosidase H-releasable oligosaccharides of this enzyme including those which are phosphorylated and involved in targeting to lysosomes. In this study, we report the characterization of the endoglycosidase H-resistant oligosaccharides which were released from β-glucuronidase with anhydrous hydrazine. Approximately 65% of the hydrazine-released oligosaccharides are of the high mannose type, with the predominant species containing 9 mannose residues. The remaining oligosaccharides appear to originate from incomplete complex oligosaccharides. Their basic structures are Man₆,₆Man₁,4GlcNAcF₁,4GlcNAcol, and Man₆,₆Man₁,4GlcNAcF₁,4GlcNAcol. One half of each species contains an additional fucose linked α1,6 to the N-acetylglucosaminol (GlcNAcol) residue. The small amount of complex oligosaccharide present bearing 1 sialic acid was heterogeneous in nature with incompletion of the nonsialylated branch. In addition, there was a minor species of high mannose-type oligosaccharide bearing 9 mannose residues with an α1,6-linked fucose on the GlcNAcol. This structure was not expected since high mannose-type oligosaccharides have been reported to not be substrates for the α1,6-fucosyl transferase.

Acid hydrolases are localized intracellularly within lysosomes. In the case of fibroblasts and presumably other cell types, transport of these hydrolases to the lysosomal compartment from their site of synthesis and/or from the extracellular milieu involves the specific recognition of high mannose-type oligosaccharides bearing 1 or 2 mannose-6-PO₄ moieties (1–10). In addition to receptor-mediated endocytosis based on recognition of mannose-6-PO₄ moieties, a receptor system is present on both alveolar macrophages and hepatic reticuloendothelial cells which is able to mediate the transport of acid hydrolases such as preputial β-glucuronidase to lysosomes based on the recognition of high mannose-type oligosaccharides and specific degradation products of complex tetra-branched oligosaccharides (11, 12). Although these distinct oligosaccharide-specific receptor systems have been extensively characterized, little information about the oligosaccharide structures present on lysosomal hydrolases has thus far been established. We have, therefore, examined the structures of all of the oligosaccharide species present on β-glucuronidase prepared from human spleen (4). In a previous study, we reported the structural analysis of the phosphorylated and nonphosphorylated high mannose species released from β-glucuronidase by endoglycosidase H digestion (13). We report here the structures of the oligosaccharides subsequently released by treatment with anhydrous hydrazine.

EXPERIMENTAL PROCEDURES AND RESULTS

Based on the mannose content/mol of β-glucuronidase monomeric subunit and the proportion of each oligosaccharide species found, the human β-glucuronidase preparation examined contains 3–4 oligosaccharide moieties/subunit (Table I). Phosphorylated oligosaccharides account for 0.3 mol of oligosaccharide/mol of β-glucuronidase subunit, indicating that as much as 30% of the β-glucuronidase monomeric species may bear a single phosphorylated oligosaccharide. Since β-glucuronidase is normally found in a tetrameric form, this would suggest that a high proportion of the tetramers contain at least 1 phosphorylated oligosaccharide moiety. It is also possible that some tetramers have multiple phosphorylated oligosaccharide moieties, while others contain no phosphate at all. Treatment of the native enzyme with endoglycosidase H results in the release of all the phosphorylated oligosaccharides and 1 additional mol of neutral high mannose-type oligosaccharide. The hydrazine-released oligosaccharides account for 2.0 mol of oligosaccharide/monomer. The high mannose oligosaccharides released by endoglycosidase H consist almost exclusively of species containing 5 and 6 mannose residues, whereas the dominant species among the endoglycosidase H-resistant oligosaccharides contains 9 mannose residues (Table I).

Several interesting features of the hydrazine-released oligosaccharides are apparent from the structures and proportions shown in Table I. Although roughly half of the oligosaccharides released by hydrazine are of the high mannose type,

1 Portions of this paper (including "Experimental Procedures," "Results," and Figs. 1–9) 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, 9520 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-670, cite the authors, and include a check or money order for $8.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
### Table I

Structures of the hydrazine-released endoglycosidase H-resistant oligosaccharides present on human β-glucuronidase

| Pool | Structure | Abbreviation | Hydrazine-released oligosaccharides |
|------|-----------|--------------|-------------------------------------|
| IX   | Man<sub>1</sub> → 6Manβ1 → 4GlcNAcβ1 → 4GlcNAcol<sup>†</sup> | M<sub>2</sub> | 6.3 |
| VIIIa | Man<sub>1</sub> → 6Manβ1 → 4GlcNAcβ1 → 4GlcNAcol | FM<sub>2</sub> | 5.8 |
| VIIIb | Man<sub>1</sub> | M<sub>3</sub> | 6.0 |
| VII  | Man<sub>1</sub> | FM<sub>3</sub> | 14.4 |
| VI′  | Man<sub>1</sub> | FM<sub>4</sub> | 1.5 |
| VIa′ | Man<sub>1</sub> | M<sub>4</sub> | 3.7 |
| VIa  | Man<sub>1</sub> | M<sub>5</sub> | 5.6 |
| VIb  | Man<sub>1</sub> | M<sub>6</sub> | 3.1 |
TABLE I (cont.)

| Pool | Structure | Abbreviation | Hydrazine-released oligosaccharides |
|------|-----------|--------------|-------------------------------------|
| VIId | Manal     | M₀          | 1.6                                 |
|      | (Manal → 2)₃ Manal |           |                                     |
|      | Manal → 4GlcNAcβ₁ → 4GlcNAcol |           |                                     |
|      | Manal     | M₁₀     | 47.4                                |
|      | (Manal → 2)₃ Manal |           |                                     |
|      | Manal → 4GlcNAcβ₁ → 4GlcNAcol |           |                                     |
|      | Manal → 2Manal |          |                                     |
|      | ± Fuc     | S₁       | 4.9                                 |
|      | Galβ₁ → 4GlcNAcβ → Manal |         |                                     |
|      | ± Fuc     | S₁       | 4.9                                 |
|      | Galβ₁ → 4GlcNAcβ → Manal |         |                                     |
|      | NANAa → Galβ₁ → 4GlcNAcβ → Manal |       |                                     |

*GlcNAcβ, N-acetylglucosaminol.

TABLE II

| Structure | Endoglycosidase released | Phosphorylated | Hydrazine released |
|-----------|--------------------------|----------------|-------------------|
| A-1       | Neutral                  |                |                   |
| M₁        | 0.04                     | 0.06           | 0.08              |
| FM₀       | 0.08                     | 0.01           | 0.03              |
| M₂        | 0.08                     | 0.01           | 0.08              |
| FM₄       | 0.07                     | 0.01           | 0.04              |
| FM₅       | 0.06                     | 0.01           | 0.04              |
| S₁        | 0.05                     | 0.01           | 0.04              |

*A-1, a single phosphate in diester linkage; A-2, a single phosphate in monoester linkage; A-3, 2 phosphates both in diester linkage. See Ref. 13 for details of structural analyses.

those with 5, 6, 7, or 8 mannose residues constitute a small proportion of the total, compared to the major species with 9 mannose residues. Oligosaccharides with 1, 2, or 3 mannose residues (M₁, FM₀, M₂, FM₄, and S₁) may have originated from incompletely processed or degraded complex oligosaccharides and account for a total of 0.78 mol of oligosaccharide/mol of β-glucuronidase subunit. The presence of a small amount of fucosylated high mannose-type oligosaccharide with 5 mannose residues was unexpected (FM₅ in Table I). Examination of the pathway for fucosylation of complex oligosaccharides has indicated that the high mannose species are not substrates for the fucosyl transferases thus far examined (14); however, oligosaccharides with the structure of M₆ apparently can become fucosylated under some circumstances.

Goldberg and Kornfeld (15) have examined glycosylation of β-glucuronidase in a murine macrophage cell line. A maximum of 30% of the β-glucuronidase subunits bears a single phosphorylated oligosaccharide which, in the majority of cases, is in the form of a phosphate in diester linkage. The phosphorylated oligosaccharides are believed to be distributed among all 3 glycosylation sites present on this glycoprotein. In addition, the neutral high mannose-type oligosaccharides at each site are predominantly composed of 9 mannose residues, whereas phosphorylated species are processed to oligosaccharides with fewer mannose residues. Their observations are similar to those we have reported here and previously (13) for human β-glucuronidase. Peptide mapping data strongly favor relatively nonspecific phosphorylation at any of the 3 possible glycosylation sites (15). Hasilik and von Figura (16) have also obtained evidence of considerable oligosaccharide heterogeneity among the glycosylation sites of cathepsin D and β-hexosaminidase.

The presence of complex oligosaccharide moieties is consistent with the binding of this and other lysosomal hydrolases by Ricin agglutinin. In addition, secreted enzymes from I-cell fibroblasts have a greater proportion of enzyme susceptible to binding by Ricin agglutinin (17). This may indicate that inhibition of transport of these enzymes to the lysosome permits the further processing of oligosaccharides with the addition of peripheral galactose moieties. Thus, it is possible that the β-glucuronidase we have characterized has incompletely synthesized complex oligosaccharides rather than partially degraded ones. We cannot differentiate these possibili-
ties with the current material. Goldberg and Kornfeld (15) also noted the presence of some complex oligosaccharide at 1 of the glycosylation sites of murine β-glucuronidase.

It is clear from this study that, in addition to the phospho-
rylated oligosaccharides, β-glucuronidase bears high man-
nose-type oligosaccharides which we have previously demon-
strated can mediate the endocytosis of glycoproteins by retic-
uloendothelial cells (12). There are, however, no structures
present which would be recognized by the hepatocyte Gal/
GalNAc-specific receptor (18). It may be that such structures
are synthesized by further processing in patients with Muco-
lipidosis II or III and could account for the relatively normal
enzyme levels found in the liver of these patients (19). Finally,
it is not yet clear which of the phosphorylated high mannose-
type oligosaccharides we and others have described are able
to mediate transport to the lysosome. Studies with the indi-
vidual characterized oligosaccharides will be required to an-
swer this question.

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chromatography described by Yaashita et al. (29). The solution was dried at pH fold molar excess of hydrazine-released oligosaccharides and a neoglycosylation digestion of microsomal endoglycosidase-R (M3). M3, Manal6 Mnahc4 GlcNAc2L GlcNAc1 and M3, Manal6 Mnahc4 GlcNAc2L GlcNAc1 were prepared by limited a-mannosidase digestion of M1 and FM3. M3 and FM3 were suspended in a 0.5 M sodium acetate, pH 5.0, prior to use. The enzyme was stored at -20°C and heated in a sealed tube for 10 hours. The reaction was terminated by addition of glacial acetic acid, and the product was passed through a Bio-Gel P-4 column (41) for 48 hr to remove peptide degradation products. The oligosaccharides were isolated by elution of the region from 2.1 to 4.1 cm from the origin with water.

Results - The preparative fractionation of the hydrazine-released oligosaccharide species on Bio-Gel P-4 is shown in Figure 2. The fractions were subjected to each neutral fraction 1.5, 5, was subsequently fractionated on MicroPor AX-5 (Figure 2). The structure of each of the oligosaccharide species thus obtained was then determined.

Figure 2: Fractionation of Neutral Species by Chromatography on Bio-Gel P-4. Fractions V, VI, VII, IX, X, XI, and XII were collected as indicated and pooled as described. The elution positions of M1, M2, FM1, FM2, FM3, and FM4 are indicated in the top panel.

Fraction 21 - The material in fraction 21 was essentially homogeneous when analyzed on MicroPor AX-5 (Fig. 2) and compared with authentic M1. Treatment with a-mannosidase (28) resulted in the release of a mannose residue (Fig. 3, Panel A). Subsequent treatment with a-fucosidase caused the release of an additional residue of mannose (Fig. 3, Panel B). Treatment with peridate followed by reduction and mild acid hydrolysis converted the oligosaccharide from which microsomal endoglycosidase-R (M3) was partially purified by the method of Catlett and Catlett (26). Endo-B-N-acetylglucosaminidase (M1), -galactosidase (M3) and -mannosidase (M2), were prepared by the method of Catlett and Catlett (26). Endo-B-N-acetylglucosaminidase (M3) and -mannosidase (M2) were used in the presence of copper chloride in 0.25 M sodium acetate, pH 5.0, for 24 hr. The reaction was terminated by addition of glacial acetic acid, and the product was passed through a Bio-Gel P-4 column for 48 hr to remove peptide degradation products. The oligosaccharides were isolated by elution of the region from 2.1 to 4.1 cm from the origin with water.

Figure 1: Preparative Fractionation on Bio-Gel P-4. Fractions V, VI, VII, VIII, IX, and X were collected as indicated and pooled as described. The elution positions of M1, M2, FM1, FM2, FM3, and FM4 are indicated in the top panel.

Fraction 21 - The material in fraction 21 was essentially homogeneous when analyzed on MicroPor AX-5 (Fig. 2) and compared with authentic M1. Treatment with a-mannosidase (28) resulted in the release of a mannose residue (Fig. 3, Panel A). Subsequent treatment with a-fucosidase caused the release of an additional residue of mannose (Fig. 3, Panel B). Treatment with peridate followed by reduction and mild acid hydrolysis converted the oligosaccharide from which microsomal endoglycosidase-R (M3) was partially purified by the method of Catlett and Catlett (26). Endo-B-N-acetylglucosaminidase (M3) and -mannosidase (M2), were prepared by the method of Catlett and Catlett (26). Endo-B-N-acetylglucosaminidase (M3) and -mannosidase (M2) were used in the presence of copper chloride in 0.25 M sodium acetate, pH 5.0, for 24 hr. The reaction was terminated by addition of glacial acetic acid, and the product was passed through a Bio-Gel P-4 column for 48 hr to remove peptide degradation products. The oligosaccharides were isolated by elution of the region from 2.1 to 4.1 cm from the origin with water.

Figure 2: Fractionation of Neutral Species by Chromatography on Bio-Gel P-4. Fractions V, VI, VII, IX, X, XI, and XII were collected as indicated and pooled as described. The elution positions of M1, M2, FM1, FM2, FM3, and FM4 are indicated in the top panel.
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Figure 1: Structural Analysis of Fraction IX. Aliquots from fraction IX were subjected to sequential exoglycosidase digestions and periodate oxidation. The products were analyzed on MicroPak AX-5.

Panel A: α-D-mannosidase (from J. B.) digestion. Panel B: a-fucosidase digestion of α-D-mannosidase product. Panel C: α-D-galactosidase (from J. B.) digestion of α-D-mannosidase, α-D-mannosidase product. Panel D: sequential digestion following removal of the α-linked mannose of standard oligosaccharides. Panel E: periodate oxidation of the α-linked mannose, α-D-mannosidase product.

This material comigrated with authentic M4 and M3 standards. Periodate oxidation of the α-linked mannose in the authentic M4 was confirmed by methylation analysis and by periodate oxidation. This was confirmed by methylation analysis and by periodate oxidation.

Figure 2: Analysis of Fraction X. This fraction was also subject to sequential exoglycosidase digestions and periodate oxidation. The products were analyzed on MicroPak AX-5.

Panel A: α-D-mannosidase (from J. B.) digestion. Panel B: a-fucosidase digestion of α-D-mannosidase product. Panel C: a-fucosidase digestion of α-D-mannosidase, α-D-mannosidase product. Panel D: sequential digestion following removal of the α-linked mannose of standard oligosaccharides. Panel E: periodate oxidation of the α-linked mannose, α-D-mannosidase product.

This material comigrated with authentic M4 and M3 standards. Periodate oxidation of the α-linked mannose in the authentic M4 was confirmed by methylation analysis and by periodate oxidation. This was confirmed by methylation analysis and by periodate oxidation.

Figure 3: Structural Analysis of Fraction IX. Aliquots from fraction IX were subjected to sequential exoglycosidase digestions and periodate oxidation. The products were analyzed on MicroPak AX-5.

Panel A: α-D-mannosidase (from J. B.) digestion. Panel B: a-fucosidase digestion of α-D-mannosidase product. Panel C: a-fucosidase digestion of α-D-mannosidase, α-D-mannosidase product. Panel D: sequential digestion following removal of the α-linked mannose of standard oligosaccharides. Panel E: periodate oxidation of the α-linked mannose, α-D-mannosidase product.

This material comigrated with authentic M4 and M3 standards. Periodate oxidation of the α-linked mannose in the authentic M4 was confirmed by methylation analysis and by periodate oxidation. This was confirmed by methylation analysis and by periodate oxidation.

Figure 4: Structural Analysis of Fraction X. Aliquots from fraction X were subjected to sequential exoglycosidase digestions and periodate oxidation. The products were analyzed on MicroPak AX-5.

Panel A: α-D-mannosidase (from J. B.) digestion. Panel B: a-fucosidase digestion of α-D-mannosidase product. Panel C: a-fucosidase digestion of α-D-mannosidase, α-D-mannosidase product. Panel D: sequential digestion following removal of the α-linked mannose of standard oligosaccharides. Panel E: periodate oxidation of the α-linked mannose, α-D-mannosidase product.

This material comigrated with authentic M4 and M3 standards. Periodate oxidation of the α-linked mannose in the authentic M4 was confirmed by methylation analysis and by periodate oxidation. This was confirmed by methylation analysis and by periodate oxidation.

Figure 5: Structural Analysis of Fraction XII. Aliquots from fraction XII were subjected to sequential exoglycosidase digestions and periodate oxidation. The products were analyzed on MicroPak AX-5.

Panel A: α-D-mannosidase (from J. B.) digestion. Panel B: a-fucosidase digestion of α-D-mannosidase product. Panel C: a-fucosidase digestion of α-D-mannosidase, α-D-mannosidase product. Panel D: sequential digestion following removal of the α-linked mannose of standard oligosaccharides. Panel E: periodate oxidation of the α-linked mannose, α-D-mannosidase product.

This material comigrated with authentic M4 and M3 standards. Periodate oxidation of the α-linked mannose in the authentic M4 was confirmed by methylation analysis and by periodate oxidation. This was confirmed by methylation analysis and by periodate oxidation.
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fraction 2 - This was the major species among the tyrosine released oligosaccharides and was homogeneous by analysis on Micropak AX-5 (Fig. 1). Digestion with α-mannosidase yielded material which comigrated with M4 (Fig. 2, Panel A) and was susceptible to sequential digestion with α-mannosidase and α-N-acetylglucosaminidase. Digestion with endo-glycosidase H-7 released N-acetylglucosaminidase (Fig. 6, Panel B). Digestion with α-L-iduronidase specific mannosidase from Asparagillus niger yielded material of 6.5 glucose equivalents on Bio Gel P-4, coinciding with authentic M6 (Fig. 2, Panel C). Methylation analysis was also consistent with the structure shown in Table I for fraction 7.

Figure 6: Structural Analyses of Fraction VII.
Analyses were performed on Micropak AX-5 (Panel A) or BioGel P-4 (Panel B and C); standards are as indicated.
Panel A: α-mannosidase digestion.
Panel B: a-mannosidase digest of α-mannosidase product.
Panel C: α-N-acetylglucosaminidase digestion of α-mannosidase, α-mannosidase product.
Panel D: α-D-mannosidase digestion of α-mannosidase, α-mannosidase product.
Panel E: endo-glycosidase-D digestion of native compound.
Panel F: perlolate oxidation of native fraction VII.

fraction 3 and 4 - The major portion of the radioactivity in fraction 3 was not identified as carbohydrate by gas chromatography or HPLC (2). Further fractionation of fraction 3 on Micropak AX-10 in 25 mM KH_{2}PO_{4}, pH 6.0, as described previously (12,20) yielded only material which comigrated with species having a single sialic acid moiety per oligosaccharide unit. Digestion with α-N-acetylglucosaminidase converted all of this material to neutral species. When examined on Bio Gel P-4 following removal of sialic acid, a heterogeneous product ranging from roughly 10 to 14 glucose equivalents was seen (Fig. 9, Panel A). This was most consistent with a branched complex oligosaccharide with incomplete branch. Sequential digestion with α-galactosidase and α-N-acetylglucosaminidase markedly reduced the heterogeneous appearance by shifting the larger species to material which comigrated with oligosaccharides in the range of 10 to 11 glucose equivalents (Fig. 9, Panels B and C). Following removal of the sialic acid, digestion with α-galactosidase and α-N-acetylglucosaminidase yielded species with equilibria on Bio Gel P-4 equal to 7.5 and 6.5 glucose equivalents (Fig. 9, Panel D). Digestion of this product with endo-glycosidase H converted (13) of the oligosaccharides to material which comigrated with Fucα1,2Galβ1,4GlcNAc and α-N-acetylglucosaminidase followed by endo-glycosidase H did not result in the production of GlcNAc or Fucα1,2Galβ1,4GlcNAc, and it appears that essentially all of the sialic acid is present in the branch arising from the α-L-iduronic acid (22). In addition, the sensitivity of the product of α-galactosidase and α-N-acetylglucosaminidase digestion to α-galactosidase (Panel D) indicates the presence of a typical core structure for complex oligosaccharides with a linked mannose residue. Finally, digestion of the product shown in Panel D with α-fucosidase yielded only GlcA, which migrates as 4 glucose equivalents (Fig. 8, Panel F) confirming the presence of α-L-iduronic acid. The range of structures present in these oligosaccharide fractions is shown in Table 1. This material was present in such a small quantity as to prevent a more detailed analysis of the heterogeneity.

Figure 7: Structural Analyses of Fraction VI Sub-Species.
Analyses were performed on Micropak AX-5; standards are as indicated.
Panel A: α-mannosidase digestion of Vla. (Identical elution profiles were obtained for VIb, VIc and VIa data not shown).
Panel B: α-N-acetylglucosaminidase digestion of Vla. The arrow at Fraction 25 indicates the position of Fm.
Panel C: α-mannosidase digestion of VIa α-mannosidase digestion product.
Panel D: endo-glycosidase H digestion of VIa.
Panel E: α-fucosidase digestion of VIa after α-mannosidase and α-N-acetylglucosaminidase digestion.

The two peaks in Panels C, B and E indicate the positions of authentic GlcA and Glc.
Figure 9: Structural Analysis of Fraction II.

Analyses were performed on Fraction II. The elution positions of glucose oligomers used as standards are indicated as V0,
V1, the void volume and V2, the included volume.
Panel A: desialylated fraction II.
Panel B: α-galactosidase digestion followed by desialylation.
Panel C: α-galactosidase and α-hexosaminidase digestion followed
by desialylation.
Panel D: digestion with α-neuraminidase, α-galactosidase, and α-
hexosaminidase.
Panel E: digestion of the product in Panel D with α-mannosidase.
Panel F: digestion of the product in Panel D with exoglycosidase D.
Panel G: digestion of the product in Panel F with α-mannosidase
and α-fucosidase.
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