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Structural assessment of β-glucuronidase carbohydrate chains by lectin affinity chromatography

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Rat liver β-glucuronidase was studied by sequential lectin affinity chromatography. β-Glucuronidase glycopeptides were obtained by extensive Pronase digestion followed by *N*-[^{14}C]acetylation and desialylation by neuraminidase treatment. According to the distribution of the radioactivity in the various fractions obtained by chromatography on different lectins, and on the assumption that all glycopeptides were acetylated to the same specific radioactivity, a relative distribution of glycan structure types is proposed. The presence of complex biantennary and oligomannose type glycans (56.8% and 42.7%, respectively) was indicated by Concanavalin A–Sepharose chromatography. *Ulex europaeus* agglutinin–agarose chromatography revealed the presence of α(1-3)linked fucose in some of the complex biantennary type glycans (16.6% of the total glycopeptides). Wheat germ agglutinin chromatography indicated that the minority (0.5%) were hybrid or poly (*N*-acetyllactosamine) type glycans. Furthermore, the absence of O-glycans, tri-, tetra- and bisected biantennary type glycans was demonstrated by analysis of Concanavalin A–Sepharose unbound fraction by chromatography on immobilized soybean agglutinin, *Ricinus communis* agglutinin and *Phaseolus vulgaris* erythroagglutinin.

Keywords: rat liver β-glucuronidase, carbohydrate structure, lectin affinity chromatography

Introduction

β-Glucuronidase (β-D-glucuronide-glucuronohydrolase EC 3.2.1.31) splits naturally occurring and artificial glucuronides into glucuronic acid and the aglycones. For this acid hydrolase dual localization in the lysosomes and microsomes [1, 2] and multiple intracellular forms are reported [3]. Recent studies have demonstrated that glycosylation of lysosomal enzymes plays an essential role in their recognition by specific receptors, intracellular segregation and targeting into lysosomes [4, 5]. For these reasons, knowledge of the primary structure of the carbohydrate chains is necessary.

Although β-glucuronidase has been isolated from rodent liver and its physicochemical properties have been described [6–11], few data are available on the carbohydrate moiety structure of this enzyme. Using lectin affinity immunoelectrophoresis, the carbohydrate structure of rat liver β-glucuronidase was assessed [12].

In order to confirm and complete previous data, we report on the qualitative assessment of glycan structure of β-glucuronidase purified by immunoaffinity chromatography from rat liver lysosomal fraction. This was performed by serial lectin affinity chromatography of glycopeptides derived from β-glucuronidase by extensive Pronase treatment.

Materials and methods

Materials

The following materials were obtained from the suppliers indicated: CNBr-activated Sepharose 4B, Protein A–Sepharose Cl-4B, Concanavalin A (Con A)–Sepharose 4B (lot No. IK 30256, 10 mg protein per ml gel) and lentil lectin (LCA)–Sepharose 4B (lot No. IH 32287, 2 mg protein per ml gel) were from Pharmacia LKB Biotechnology AB, Uppsala, Sweden; *Phaseolus vulgaris* erythroagglutinin (E-PHA)–agarose (lot No. 48F4014, 2.8 mg protein per ml gel), *Ricinus communis* agglutinin I (RCA_I)–agarose (lot No. 69F40271, 2.3 mg protein per ml gel), *Triticum vulgaris* agglutinin (WGA)–agarose (lot No. 48F4027, 5 mg protein per ml gel), *Glycine max* agglutinin (SBA)–agarose (lot No. 70H4048, 4 mg protein per ml gel), *Ulex europaeus* agglutinin (UEA)–agarose (lot No. 69F4032, 3.5 mg protein per ml gel), phenolphthalein glucuronic acid, and neuraminidase (type X from *Clostridium perfringens*) (lot No. 76F9585, 1 U per 2.6 ml agarose suspension) were from Sigma Chemical Co., St Louis, MO, USA; Pronase E (4 mln PU g^{-1}) was from Merck, Darmstadt, Germany; Bio-Gel

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P-2 (200–400 mesh) was from Bio-Rad Laboratories, Richmond, CA, USA and [1-^{14}C]acetic anhydride (31 mCi $mmol^{-1}$) from Amersham International, Amersham, UK. All other chemicals used were of the highest grade available and were obtained from commercial sources.

Isolation of rat liver β-glucuronidase

Rat liver β-glucuronidase from crude lysosomal extract obtained according to the method of De Duve *et al.* [1] was isolated by immunoaffinity chromatography. Antibodies against β-glucuronidase were isolated from 20 ml (86.8 mg total protein) of rabbit antiserum by chromatography on Protein A–Sepharose Cl 4B column (1 cm × 10 cm) in 0.1 M sodium phosphate buffer, pH 7.0 [13]. Affinity purified antibodies (8.9 mg) were dialysed against 0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.3, and then coupled to CNBr-activated Sepharose 4B (2.5 g) [14]. Affinity chromatography of β-glucuronidase was performed in 20 mM Tris-HCl buffer, pH 7.4, containing 0.2 M NaCl on antibodies–Sepharose 4B column (1 cm × 11 cm). The column was washed with starting buffer, and β-glucuronidase was then eluted with 0.2 M glycine-HCl buffer, pH 3.0. The pH in collected fractions was immediately neutralized by addition of 0.7% Tris. Fractions containing enzymatic activity were pooled, concentrated and examined for purity by 7.5% polyacrylamide gel electrophoresis [15]. To remove minor contaminations, preparative electrophoresis was performed as described previously [12].

Preparation of N-[^{14}C]acetylated glycopeptides from rat liver β-glucuronidase

Glycopeptides were prepared by extensive digestion of 6 mg β-glucuronidase with 2% (w/w) Pronase in 0.1 M Tris-HCl buffer, pH 8.0, 1 mM $CaCl_2$, 0.2% Triton X-100 at 37 °C for 72 h under a toluene atmosphere. The Pronase was preincubated for 1 h at 37 °C in order to inactivate any possible endogenous glycosidase impurity. Additional amounts of Pronase (2% w/w) were added at 12 h intervals. The digestion was stopped by heating the incubation mixture at 100 °C for 10 min, followed by centrifugation at 15 000 × g for 5 min to remove any insoluble material. The supernatant was desalted on Bio-Gel P-2 (1.6 cm × 100 cm) in water, and glycopeptides were monitored by the phenol/H_2SO_4 reaction [16]. Fractions containing glycopeptides were concentrated by vacuum evaporation, and the glycopeptides of β-glucuronidase were acetylated with 8.1 μmol [^{14}C]acetic anhydride (31 mCi $mmol^{-1}$) in 2 ml 60 mM $NaHCO_3$ at room temperature for 2 h [17]. The incubation mixture was subjected to gel filtration on a column (1.6 cm × 60 cm) of Bio-Gel P-2 in water. Fractions containing the ^{14}C labelled glycopeptides were pooled and concentrated.

Lectin–agarose (Sepharose) affinity chromatography

Glycopeptides were further analysed by serial lectin–agarose (Sepharose) chromatography. The radioactivity in aliquots from each lectin column fraction was determined in an LKB Wallac 1211 Rackbeta liquid scintillation counter. Appropriate fractions were pooled and desalted by gel filtration in water on a column of Bio-Gel P-2 (1.6 cm × 100 cm) and then concentrated by vacuum evaporation. After reconstitution in the appropriate buffer, the glycopeptide fractions were subjected to the subsequent lectin affinity chromatography.

Con A–Sepharose chromatography was performed on a 0.5 ml column (0.6 cm × 1.8 cm; 1 ml min^{-1}; 2 ml fractions; 20 °C) in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM $CaCl_2$, $MnCl_2$ as starting buffer [18]. After application of sample (0.5 ml) in starting buffer, the material not bound by the lectin (C_0) was eluted with 18 ml of the same buffer. Specific elution of bound material was performed with 14 ml 0.01 M methyl-α-mannopyranoside (C_1) followed by 14 ml 0.5 M methyl-α-mannopyranoside in starting buffer (C_2).

RCA_I-agarose chromatography was performed using a 1.5 ml column (0.6 cm × 5.3 cm; 5 ml h^{-1}; 1 ml fractions; 20 °C) in 0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl as starting buffer [18]. After application, sample (0.5 ml) (C_0) was allowed to interact with the lectin for 2 h. Elution was performed with 17 ml starting buffer (unbound fraction C_0R_0) and then with 13 ml 0.3 M galactose in starting buffer (bound fraction C_0R_1).

SBA–agarose chromatography was performed in a 1 ml column (0.6 cm × 3.5 cm; 5 ml h^{-1}; 1 ml fractions; 20 °C) in 0.01 M Tris-HCl, pH 7.6, 0.15 M NaCl as starting buffer. After application, sample (C_0R_0, 0.5 ml) was allowed to interact with the lectin for 2 h. Unbound fraction ($C_0R_0S_0$) was eluted with 17 ml starting buffer, then elution was continued with 0.25 M sodium tetraborate in starting buffer (bound fraction $C_0R_0S_1$).

E-PHA–agarose chromatography was performed using a 1 ml column (0.35 cm × 10 cm; 5 ml h^{-1}; 0.06 ml fractions; 20 °C) with 6.7 mM KH_2PO_4, pH 7.4, 0.15 M NaCl as starting buffer [19]. After application, sample ($C_0R_0S_0$, 0.5 ml) was allowed to interact with the lectin for 1.5 h. Then the column was eluted with 8 ml starting buffer ($C_0R_0S_0E_0$).

WGA–agarose chromatography was performed using a 1 ml column (0.6 cm × 3.5 cm; 10 ml h^{-1}; 1 ml fractions; 20 °C) with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl as starting buffer [19]. After application of samples (C_1 or C_2L_0, 0.5 ml), elution was performed with 10 ml starting buffer (unbound fractions C_1W_0 or $C_2L_0W_0$, respectively) followed by specific elution with 5 ml 0.2 M *N*-acetylglucosamine in starting buffer (bound fraction C_1W_1).

LCA–Sepharose chromatography was performed using a 1 ml column (0.6 cm × 3.5 cm; 1 ml min^{-1}; 1 ml fractions;

20 °C) with 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM $CaCl_2$, $MgCl_2$, as starting buffer [18]. After application of samples (0.5 ml) (C_1W_0 or C_2) elution was performed with 20 ml starting buffer (unbound fractions $C_1W_0L_0$ or C_2L_0, respectively) followed by specific elution with 20 ml 0.5 M methyl-α-mannopyranoside in starting buffer (bound fraction $C_1W_0L_1$).

UEA–Agarose chromatography was performed using a 1 ml column (0.6 cm × 3.5 cm; 5 ml h^{-1}; 1 ml fractions; 20 °C) with 0.01 M sodium phosphate, pH 7.2, 0.15 M NaCl as starting buffer. After application, sample ($C_1W_0L_0$, 0.5 ml) was allowed to interact with the lectin for 1 h. Then elution was performed with 15 ml starting buffer (unbound fraction $C_1W_0L_0U_0$) followed by specific elution with 15 ml 0.3 M L-fucose in starting buffer (bound fraction $C_1W_0L_0U_1$).

Percentages were calculated by taking the radioactivity of the total eluate from the various lectin affinity columns as 100%.

Other methods

β-Glucuronidase was assayed [20] as described previously [12]. Protein was determined by a dye-binding assay method [21] using bovine serum albumin as standard. Samples for neuraminidase digestion were adjusted to 100 mM sodium citrate:citric acid, pH 5.0, and then incubated for 48 h at 37 °C with 2.6 ml suspension of neuraminidase attached to beaded agarose (1U).

Results and discussion

Glycoproteins may possess Asn-linked (N-linked) or Ser/Thr-linked (O-linked) oligosaccharides or a mixture of both types. N-Glycans are generally classified as oligomannose, hybrid, or complex types [22, 23]. In this study, Pronase digested rat liver lysosomal β-glucuronidase was subjected to sequential lectin affinity chromatography, on seven immobilized lectins (Con A, LCA, WGA, RCA_I, SBA, E-PHA and UEA) in order to assess its glycan structure. The procedure of chromatography is shown in Fig. 1. The lines running between different chromatographic profiles (Fig. 1) indicate the sequence of steps.

Con A–Sepharose was used as a first step. Under conditions described in the Materials and methods section this column binds biantennary complex, hybrid, and oligomannose type glycopeptides. The lectin also binds biantennary complex fucosylated and oligomannose phosphorylated type glycans. This lectin does not bind tri- and tetraantennary complex, bisected biantennary complex and O-linked glycans [18]. Differential elution with methyl-α-mannopyranoside enables separation of biantennary complex and hybrid from oligomannose type glycans. β-Glucuronidase glycopeptides were separated by this column in three fractions: not bound C_0, 0% (this fraction did not contain any glycopeptides; radioactivity represented only acetylated amino acids and peptides) bound C_1, 57.3% and C_2, 42.7%. Fraction C_2 was bound more strongly than C_1 (eluted with 500 mM and 10 mM methyl-α-mannopyranoside, respectively) indicating the presence of biantennary complex and/or biantennary poly(*N*-acetyllactosamine) and/or hybrid type glycans in C_1 and oligomannose and/or hybrid in C_2. However, asialo, agalacto biantennary complex type glycans might also be present in C_2 (18). The fractions C_0, C_1, and C_2 were treated with neuraminidase under conditions described in the Materials and methods section, and further analysed using subsequent columns.

Tri- and tetraantennary complex type glycans containing *N*-acetyllactosamine sequences were not detected in C_0 since no radioactivity was retained by RCA_I. This lectin under the conditions used binds terminal *N*-acetyllactosamine sequences present in complex and bisected complex type glycans [18]. The radioactivity present in fraction not bound to RCA_I (C_0R_0) was sequentially subjected to chromatography on SBA– and E-PHA–agarose. The lack of reaction with SBA–agarose ($C_0R_0S_0$) indicated the absence of galactose or *N*-acetylgalactosamine residues (O-linked glycans) [24, 25]. According to the specificity of E-PHA, this lectin binds bisected biantennary complex type glycans containing outer galactose residues [18, 26]. The absence of retained radioactivity on this column was observed, indicating lack of the above mentioned structures.

The radioactivity present in C_1 (after desialylation) was then subjected to sequential chromatography on WGA–agarose, LCA–Sepharose and UEA–agarose. Under the conditions used, WGA–agarose interacts with bisected hybrid and, to a lesser extent, with bisected biantennary complex type glycans. The GlcNAcβ(1-4)Manβ(1-4)-GlcNAc-β(1-4)GlcNAc-β(1-*N*)Asn sequence represents the structural determinant required by WGA [24, 26]. Only 0.5% of the total radioactivity (total means $C_1 + C_2$) was bound to WGA and eluted with 0.2 M *N*-acetylglucosamine, which indicates the presence of bisected hybrid type glycans. The fraction unbound on WGA–agarose (C_1W_0) was examined on LCA–Sepharose. This lectin interacts with tri- and biantennary complex type glycans possessing an L-fucose residue at the C-6 position of the innermost *N*-acetylglucosamine. LCA recognizes also the same structure containing a bisecting *N*-acetylglucosamine residue [18, 24, 26]. The LCA–Sepharose bound fraction ($C_1W_0L_1$) represents 0.4% of the total radioactivity, indicating that only a small part of biantennary type glycans contains a fucose residue α(1-6) linked to the inner core. The LCA–Sepharose unbound fraction ($C_1W_0L_0$) was separated on UEA–Agarose. Immobilized UEA reacts with fucose residues in an external position on a glycan whereas it has no affinity for fucosylated complex type glycans. According to the specificity of UEA, this lectin recognizes with high affinity complex type glycans containing a fucose residue α(1-3) linked to *N*-acetylglucosamine or a fucose residue α(1-2) linked to galactose [24, 27]. Two fractions were

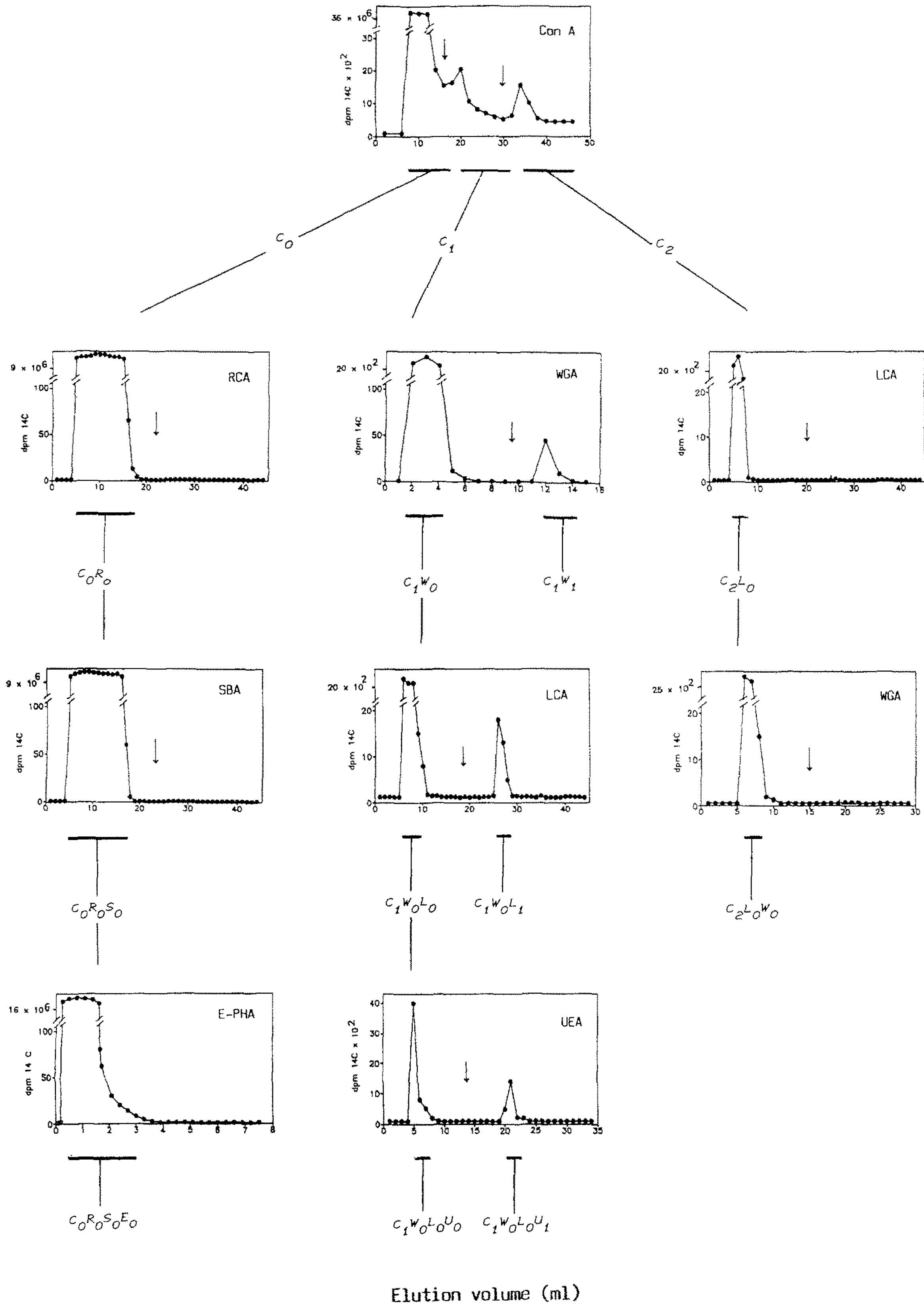

Figure 1. Lectin affinity chromatography of N-[^{14}C] acetylated β-glucuronidase glycopeptides. The fractions were prepared as described in the Materials and methods section. The lines running between different chromatographic profiles indicate the sequence of steps. The arrows within the graphs indicate the points at which eluents containing haptenic sugars were applied. Horizontal bars indicate fractions which were collected.

Table 1. Tentative assignment of oligosaccharide structures to β-glucuronidase. The values, given in % were calculated from the fractionation of the [^{14}C]acetylated glycopeptides on the immobilized lectins as indicated in Fig. 1, on the assumption that all glycopeptides have the same specific radioactivity.

Fraction	*Relative distribution of radioactivity (%) in fractions*	*Tentative assignment of glycan structure types*	(%)
$C_1W_0L_0U_0$	39.8	complex bi-antennary	39.8
$C_1W_0L_0U_1$ $C_1W_0L_1$	16.6 0.4	complex bi-antennary fucosylated	17
C_1W_1	0.5	hybrid, poly(*N*-acetyllactosamine)	0.5
$C_2L_0W_0$	42.7	oligomannose	42.7

obtained, unbound ($C_1W_0L_0U_0$) and bound ($C_1W_0L_0U_1$ eluted with 0.3 M L-fucose), comprising 39.8% and 16.6% of the total radioactivity ($C_1 + C_2$), respectively. This result suggested that the unbound fraction consisted of complex biantennary type glycans while the bound fraction contained fucosylated complex biantennary type glycans.

In the case when the C_1 fraction (before neuraminidase treatment) was used, all the radioactivity bound to WGA–agarose (data not shown).

The radioactivity present in C_2 was analysed by sequential chromatography on LCA–Sepharose and WGA–agarose. Both separations resulted in unbound fractions (C_2L_0, $C_2L_0W_0$, respectively), indicating the presence of oligomannose type glycans only (42.7% of total radioactivity) in this fraction.

According to the distribution of radioactivity in the various fractions, the relative contents of glycan structure types of β-glucuronidase is proposed as given in Table 1. Complex biantennary and oligomannose type glycans are present in about equal amounts, 56.8% and 42.7%, respectively. Among complex biantennary type glycans up to 17% may be fucosylated. A minority (0.5%) represents hybrid or poly (*N*-acetyllactosamine) type structures. We have already investigated the glycan structure of native, nondigested β-glucuronidase by lectin affinity immunoelectrophoresis [12]. This method revealed the presence of oligomannose, hybrid and complex biantennary type glycans and absence of bisected biantennary, tri-, and tetraantennary complex type glycans. The presence of fucosylated glycans was revealed by strong reaction with *Lotus tetragonolobus* lectin. However, sequential lectin chromatography of labelled glycopeptides allowed determination of the relative distribution of different types of glycans. Digestion of rat liver β-glucuronidase with endoglycosidase F, followed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis decreased the molecular weight by 6000, suggesting the presence of three glycosylation sites per β-glucuronidase subunit [11]. Analysis of proteins glycosylated at multiple sequons is difficult due to the possible diversity of N-glycan structures at a single glycosylated sequon. In this respect, characterization of different type glycans at each isolated individual sequon has to be established.

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References

1. De Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F (1955) *Biochem. J* **60**:604–17.
2. Paigen K (1961) *Expt Cell Res* **25**:286–301.
3. Swank RT, Paigen K (1973) *J Mol Biol* **77**:371–89.
4. Kornfeld R, Kornfeld S (1985) *Ann Rev Biochem* **54**:631–64.
5. Von Figura K, Hasilik A (1986) *Ann Rev Biochem* **55**:167–93.
6. Owens JW, Gammon KL, Stahl PD (1975) *Arch Biochem Biophys* **166**:258–72.
7. Tomino S, Paigen K (1975) *J Biol Chem* **250**:8503–9.
8. Owens JW, Stahl P (1976) *Biochim Biophys Acta* **438**:474–86.
9. Goldberg DE, Kornfeld S (1981) *J Biol Chem* **256**: 13060–67.
10. Swank RT, Pfister K, Miller D, Chapman V (1986) *Biochem J* **240**:445–54.
11. Powell PP, Kyle JW, Miller RD, Pantano J, Grubb JH, Sly WS (1988) *Biochem J* **250**:547–55.
12. Wójczyk B, Hoja D, Lityńska A (1991) *Glycoconjugate J* **8**:340–49.
13. Chenais F, Virella G, Patrick CC (1977) *J Immunol Methods* **18**:183–92.
14. *Affinity Chromatography: Principles and Methods*, p 15. Uppsala: Pharmacia Fine Chemicals (1979).
15. Davis BJ (1964) *Ann NY Acad Sci* **121**:321–49.

16. Du Bois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) *Anal Chem.* **28**:350–56.
17. Koide N, Muramatsu T (1974) *J Biol Chem* **249**:4897–904.
18. Merkle RK, Cummings RD (1987) *Methods Enzymol* **138**:232–59.
19. Bierhuizen MAF, Hansson M, Odin P, Debray H, Öbrink B, Van Dijk W (1989) *Glycoconjugate J* **6**:195–208.
20. Barrett AJ, Heath MF (1977) In *Lysosomes: A Laboratory Handbook* (Dingle JT, ed.) pp 118–20. Amsterdam: Elsevier.
21. Bradford MM (1976) *Anal Biochem* **272**:248–54.
22. Montreuil J, Bouquelet S, Debray H, Furnet B, Spik G, Strecker G (1986) In *Carbohydrate Analysis a Practical Approach* (Chaplin MF, Kennedy JF, eds) pp 142–68. Oxford: IRL Press.
23. Kornfeld R, Kornfeld S (1980) In *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz WJ, ed.) pp 1–34. New York: Plenum Press.
24. Debray H, Decout D, Strecker G, Spik G, Montreuil J (1981) *Eur J Biochem* **117**:41–55.
25. Wu AM, Sugii S, Herp A (1985) In *The Molecular Immunology of Complex Carbohydrates* (Wu A, Adams LG, eds) pp 819–47. New York: Plenum Press.
26. Osawa T, Tsuji T (1987) *Ann Rev Biochem* **56**:21–41.
27. Debray H, Pierce-Cretel A, Spik G, Montreuil J (1983) In *Lectins—Biology, Biochemistry, Clinical Biochemistry* (Bøg-Hansen TC, Spengler GA, eds) pp 335–50. Berlin–New York: Walter de Gruyter.