Journal of Chromatography B, 755 (2001) 173–183

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

# Affinity chromatography of branched oligosaccharides in rat liver β-glucuronidase

Dorota Hoja-Łukowicz[a,*], Anna Lityńska[a], Bogusław S. Wójczyk[b]

[a]Laboratory for Biostructural Research, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, 6 Ingardena Street, 30060 Kraków, Poland

[b]Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA

Received 30 May 2000; received in revised form 19 January 2001; accepted 19 January 2001

**Abstract**

Rat liver microsomal and lysosomal β-glucuronidase-derived glycopeptides were obtained by extensive Pronase digestion followed by *N*-[$^{14}$C]acetylation and desialylation by neuraminidase treatment. These glycopeptides were studied by sequential chromatography on lectin-affinity columns such as concanavalin A, lentil lectin, *Phaseolus vulgaris* erythroagglutinin, *Ricinus communis* agglutinin I, *Triticum vulgaris* agglutinin, *Glycine max* agglutinin and *Ulex europaeus* agglutinin. Using serial lectin affinity chromatography approach combined with neuraminidase treatment allowed us to show the unexpected presence of complex tri- and/or tetraantennary type glycans (40.8 and 17.0% for microsomal and lysosomal enzyme, respectively). Moreover, the application of neuraminidase treatment revealed that complex biantennary type glycans, present on lysosomal β-glucuronidase, are almost fully sialylated while the same type of glycans present on microsomal enzyme do not contain sialic acid. Furthermore, the results obtained confirmed that microsomal and lysosomal β-glucuronidases possess high mannose and/or hybrid type glycans (19.6 and 36.6%, respectively), and complex biantennary type glycans (38.9 and 46.4%, respectively). © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Oligosaccharides; β-Glucuronidase

## 1. Introduction

β-Glucuronidase (EC 3.2.1.31) is an acid hydrolase typically found in lysosomes. However, unlike most other lysosomal enzymes, β-glucuronidase displays a unique subcellular distribution and is associated with both the liver lysosomal and microsomal fractions [1]. Some 30–50% of rat liver β-glucuronidase activity is localised in the microsomal vesicles and the rest of it is present in lysosomes [2].

Rat liver β-glucuronidase is composed of four identical subunits, held together by non-covalent forces [3]. Individual subunits are synthesised on membrane-bound ribosomes and translocated into the lumen of the ER, where signal sequence cleavage, *N*-glycosylation, disulfide bound formation and tetramerization occurs. After signal sequence cleavage, the precursor of β-glucuronidase is formed. In endosomes, β-glucuronidase precursor undergoes a processing event, where C-terminal peptide (3000

*Corresponding author. Tel.: +48-12-633-6377, ext. 2405; fax: +48-12-634-3716.

*E-mail address:* hoja@zuk.iz.uj.edu.pl (D. Hoja-Łukowicz).

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(01)00057-3

rel. mol. mass) is being cleaved [2]. The precursor form corresponding to microsomal isoenzyme with a 78 000–75 000 rel. mol. mass of subunit, and mature form corresponding to lysosomal isoenzyme with a 75 000–73 000 rel. mol. mass of subunit have been described [4]. Some 60% of rat liver microsomal enzyme is held in microsomal vesicles by interaction with egasyn (five to ten molecules of egasyn per one molecule of β-glucuronidase), as a complex with high rel. mol. mass (form M). The remaining 40% of this enzyme, form X, does not create a complex with egasyn [2,4–6]. Microsomal and lysosomal β-glucuronidases are glycoproteins, encoded by the same gene. They are catalytically and immunologically identical, and have similar molecular properties though they are electrophoretically distinct [3,7,8], and differ somewhat in amino acids and carbohydrate compositions [9]. Each subunit of β-glucuronidase possesses four potential glycosylation sites but only three are glycosylated [10].

Despite numerous studies on rat liver microsomal and lysosomal β-glucuronidases, the structure of their carbohydrate chains has not been clarified [9,11–15]. It has been established that both enzymes possess high-mannose type asparagine linked sugar chains [12]. Moreover, the presence of minor *N*-acetyllactosamine type glycans besides the oligomannoside type glycans for lysosomal enzyme was also reported [13]. Recently, the presence of oligomannoside type glycans, minor hybrid or poly(*N*-acetyllactosamine) type glycans, as well as complex biantennary type glycans, was reported for rat liver lysosomal enzyme [14].

In order to complete previous data, we report the qualitative assessment of glycan structure of β-glucuronidase purified by immunoaffinity chromatography from rat liver microsomal and lysosomal fraction. This was performed by serial lectin affinity chromatography of glycopeptides derived from β-glucuronidase by extensive Pronase digestion. Binding specificities of used lectins have been reported by others authors and are summarised in Table 1. Surprisingly, we found that microsomal and lysosomal β-glucuronidases possess not only previously reported core and arm fucosylated complex biantennary type, oligomannosidic, and hybrid type glycans [14], but also tri- and/or tetraantennary type glycans.

## 2. Experimental

### 2.1. Reagents

The following reagents were purchased: concanavalin A (Con A)-Sepharose 4B (lot No. IK 30256, 10 mg protein/ml gel) and lentil lectin (LCA)-Sepharose 4B (lot No. IH 32287, 2 mg protein/1 ml gel) were from Pharmacia LKB Biotechnology, Uppsala, Sweden. *P. vulgaris* erythroagglutinin ($E_4$-PHA)-agarose (lot No. 48F4014, 2.8 mg protein/ml gel), *R. communis* agglutinin I (RCA I)-agarose (lot No. 69F40271, 2.3 mg protein/ml gel), *T. vulgaris* agglutinin (WGA)-agarose (lot No. 48F4027, 5 mg protein/ml gel), *G. max* agglutinin (SBA)-agarose (lot No. 70H4048, 4 mg protein/ml gel), *U. europaeus* agglutinin (UEA)-agarose (lot No. 69F4032, 3.5 mg protein/ml gel), phenolphthalein glucuronic acid, and neuraminidase (type X from *Clostridium perfringens*) were from Sigma, St Louis, MO, USA. Pronase E (4 mln PU/g) was from Merck, Darmstadt, Germany; Bio-Gel P-2 (200–400 mesh) was from Bio-Rad Laboratories, Richmond, CA, USA and [1-$^{14}$C]acetic anhydride (31 mCi/mmol) was from Amersham International, Amersham, UK. All other chemicals used were of analytical grade and were obtained from commercial sources.

### 2.2. Samples

A total of 100 male rats (3 months old) fasted for 24 h were killed by decapitation. The livers were quickly removed and chilled on ice. All subsequent operations were carried out at 4°C. A mitochondrial-lysosomal (ML) fraction was obtained according to the method of De Duve et al. [1]. A microsomal (M) fraction was obtained from post ML fraction supernatant, using the low ion-strength buffers and calcium-precipitation technique according to the method of Owens et al. [15]. The purity of both fractions was examined by electron microscopy technique according to [16,17].

### 2.3. Extraction of the M and ML fractions

Lysosomal fraction pellets and washed microsomes were suspended in 200 m*M* Tris–HCl, pH

Table 1
Sugar binding specificities of immobilised lectins used for affinity chromatography of glycopeptides

| Name of lectin affinity column | Hapten sugars used for elution | Lectin specificity | Refs. |
|---|---|---|---|
| Con A-Sepharose | 10 m*M* and 500 m*M* Methyl-α-manno-pyranoside | Biantennary type glycopeptides bind and are eluted with low hapten concentration. High mannose/hybrid type glycopeptides bind and are eluted with high hapten concentration. | [19,22,27,29,31,41] |
| RCA I-agarose | 300 m*M* Galactose | Glycopeptides with terminal galactosyl residues linked β1–4 bind with high affinity; using retardation chromatography, glycopeptides can be separated depending on the number or positions of these residues. | [19,22,23,27,29,33,34] |
| SBA-agarose | 250 m*M* Sodium tetraborate | Only GalNAcα1-Ser/Thr glycopeptide binds with high affinity; Galα1-Ser/Thr glycopeptide binds with low affinity. | [22,27] |
| $E_4$-PHA-agarose | See lectin specificity | Only certain bi- and triantennary type glycopeptides with outer galactose residues bind; glycopeptide elution is retarded and no hapten is needed. | [19,24,29,36] |
| WGA-agarose | 200 mM *N*-Acetylglucosamine | Bisected hybrid type glycopeptides bind with high affinity. *N*-Acetyllactosamine type glycopeptides present weaker affinity. Glycopeptides with a high density of terminal non-reducing *N*-acetylglucosamine or *N*-acetylneuraminic acid residues interact. | [19,25,27,33,39,40] |
| LCA-Sepharose | 500 m*M* Methyl-α-mannopyranoside | Only certain bi- and triantennary type glycopeptides that contain a core fucose residue bind. | [22,27,33,42] |
| UEA-agarose | 300 m*M* L-Fucose | Outer fucose residue is required. | [22,29,33,43] |

7.0, 0.15 *M* NaCl containing 0.2% Triton X-100, and briefly vortex-mixed. Then the suspensions were frozen and thawed three times to ensure the rupture of membranes and centrifuged at 105 000 *g* for 60 min.

### *2.4. Immunoaffinity chromatography*

Both β-glucuronidases were isolated from obtained extracts by immunoaffinity chromatography as described by Wójczyk et al. [14]. Fractions containing enzymatic activity were pooled together, concentrated and desalted using Centriprep 30 Clear devices (Centriprep Starter Kit, Amicon, Beverly, MA, USA). The resulting solutions were washed with 15 ml of distilled water and then 15 ml of 5 m*M* $(NH_4)_2CO_3$, concentrated as before and lyophilised to dryness.

### *2.5. Preparative electrophoresis*

Both isoenzymes of β-glucuronidase were purified by preparative gel electrophoresis (70×75×1 mm) in 5 m*M* Tris–glycine buffer, pH 8.6, under non-denaturing conditions, using 7.5% gel and constant voltage (200 V). Immunopurified β-glucuronidase (1 mg of total protein in 200 μl of above buffer) was loaded into a single well (55×15×1 mm). After electrophoresis thin gel stripes from both sides of gel were excised and double stained with phenolphthalein glucuronic acid to reveal localisation of enzymatic activity, and then with CBB R-250 to reveal protein bands. The part of the remaining gel corresponding to protein band and enzymatic activity was cut out, homogenised, and β-glucuronidase was recovered by elution with 5 m*M* Tris–glycine buffer, pH 8.6. The homogeneity of enzymes was confirmed by SDS–PAGE electrophoresis according to Laem-

mli [18] using 4% stacking gel and 12.6% separating gel.

### 2.6. Preparation of N-[$^{14}$C]acetylated glycopeptides from rat liver microsomal and lysosomal β-glucuronidases

Glycopeptides were prepared by extensive digestion of 3 mg microsomal and 6 mg lysosomal β-glucuronidase with Pronase according to the procedure described previously [14]. Microsomal and lysosomal glycopeptides were acetylated with 2.6 and 5.5 μmol [$^{14}$C]acetic anhydride (31 mCi/mmol) in 1 and 2 ml of 60 m*M* $NaHCO_3$ at room temperature for 2 h, respectively [19]. The labelling mixtures were desalted on a Bio-Gel P-2 column (60×1.6 cm) in water. Fractions containing the $^{14}$C-labelled glycopeptides were pooled and concentrated.

### 2.7. Serial lectin affinity column chromatography

The mixtures of radioactive 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 1209 Rackbeta liquid scintillation counter (Pharmacia, Turku, Finland). Appropriate fractions were pooled and desalted by gel filtration in water on a column of Bio-Gel P-2 (100×1.6 cm) and then concentrated by vacuum evaporation. After dissolving in the appropriate buffer, the glycopeptide fractions were subjected to the subsequent lectin affinity chromatography. The total amount of loaded glycopeptides did not exceed the column capacities. If unbound fractions were loaded again on fresh lectin column, no bound material was detected. Similarly, if the bound fractions were rechromatographed, no unbound material was found, and bound material was eluted in the same proportion as the initial one. All fractionations were conducted at 20°C. The chromatographic procedures on Con A-Sepharose (1.8×0.6 cm), $E_4$-PHA-agarose (10×0.35 cm) and LCA-Sepharose (3.5×0.6 cm) were performed in the way previously described [14]. The chromatography of glycopeptides on RCA I-agarose (5.3×0.6 cm), SBA-agarose (3.5×0.6 cm), WGA-agarose (3.5×0.6 cm) and UEA-agarose (3.5×0.6 cm) was carried out as described previously [14] with slight modification. Usually, a 500-μl sample was loaded on a column previously equilibrated with appropriate starting buffer, then the column was washed with a starting buffer to separate an unbound fraction, and then with a specific eluent to separate bound material. The separation on RCA I-agarose was performed using 17 ml of starting buffer (unbound fraction), followed with an additional 10 and 7 ml of starting buffer (retarded fractions for microsomal and lysosomal glycopeptides, respectively), and then 20 and 13 ml of 300 m*M* galactose in starting buffer (bound fractions for microsomal and lysosomal glycopeptides, respectively). The elution on SBA-agarose was performed with 50 ml of starting buffer (unbound fraction) and with 30 ml of 250 m*M* sodium tetraborate in starting buffer (bound fraction). The elution on WGA-agarose was performed with 20 ml of starting buffer (unbound fraction) and with 10 ml of 200 m*M* *N*-acetylglucosamine in starting buffer (bound fraction). The elution on UEA-agarose was carried out with 30 ml of starting buffer (unbound fraction) and with 30 ml of 300 m*M* L-fucose in starting buffer (bound fraction).

Percentages were calculated by taking the radioactivity of the total eluate from the various lectin-agarose columns as 100%. The recovery of the radioactivity on the various columns routinely was over 90%.

### 2.8. Other methods

β-Glucuronidase activity was assayed as described previously [20]. Protein was determined by a dye-binding assay method [21] using bovine serum albumin as standard. Neuraminidase digestion was performed as described previously [14].

## 3. Results

The general strategy employed in this study is outlined in Fig. 1.

### 3.1. Isolation and purification of microsomal and lysosomal β-glucuronidases

The combination of two standard techniques of isolation of ML and M fractions [1,15] allowed us to isolate and then purify both isoenzymes from the

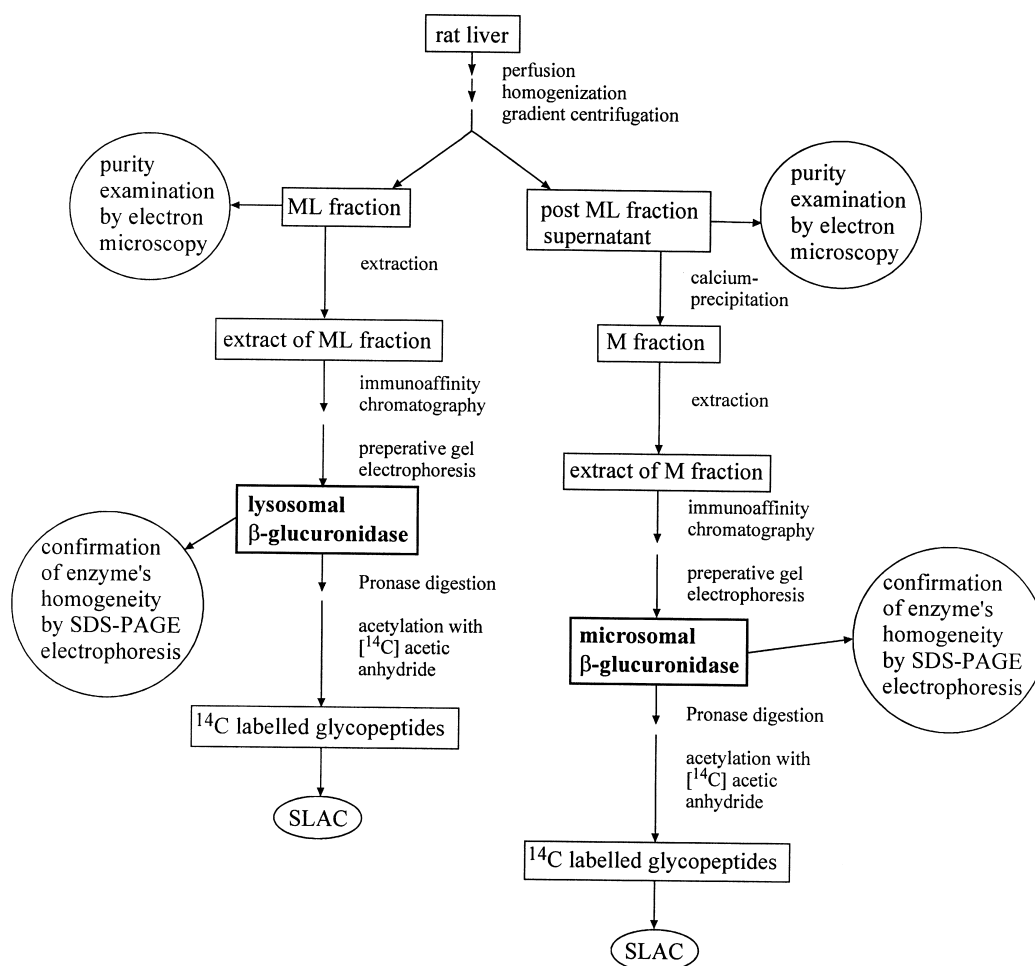


Fig. 1. General strategy for the purification and preparation steps of rat liver β-glucuronidase glycopeptides. $^{14}$C-labelled glycopeptides obtained from microsomal and lysosomal β-glucuronidase were fractionated by sequential lectin affinity chromatography according to the procedure visualised in Figs. 2 and 3, respectively.

same part of rat liver homogenate. The ML fraction was microsome-free and the M fraction showed very few lysosome-like structures when examined by electron microscopy (data not shown).

The isolation of both isoenzymes was achieved by using a lysosomal β-glucuronidase-specific antibody-Sepharose column according to Wójczyk et al. [14]. This immunoaffinity column routinely provided a purification of ~80–100-fold with a recovery of 60–80%. Purity examination of isolated isoenzymes by gel electrophoresis under non-denaturing conditions revealed minor contaminations (data not shown). To improve the purification, a preparative gel electrophoresis was performed with recovery of 60%. SDS–PAGE gel electrophoresis of highly purified isoenzymes revealed that both microsomal and lysosomal β-glucuronidases migrated as single bands with mobilities corresponding to subunit rel. mol. mass of 78 000 and 75 000, respectively (data not shown). The results obtained are in agreement with the previously reported data [9].

### *3.2. Lectin affinity chromatography*

Both rat liver microsomal and lysosomal β-glucuronidase glycopeptides, released by Pronase digestion, followed by labelling with [1-$^{14}$C]acetic anhydride, were separated by sequential lectin affinity

chromatography on seven immobilised lectins: Con A, LCA, WGA, RCA I, SBA, $E_4$-PHA and UEA.

Since the presence of sialic acid substitution on galactose residues abolishes the interaction of glycopeptides with RCA I-Sepharose, SBA-agarose and $E_4$-PHA-agarose [22–24], both A fractions (glycopeptides unbound to Con A-Sepharose column) were treated with neuraminidase (A′) before subsequent analysis (Figs. 2 and 3).

The total radioactivity of B fraction (glycopeptides weakly bound to Con A-Sepharose column) originated from lysosomal β-glucuronidase was bound to the WGA-agarose column (Fig. 3). Since the glycopeptides with high density of terminal *N*-acetylneuraminic acid residues interact with WGA (''avidity effect'') [25], this fraction was digested with neuraminidase (type X from *C. perfringens*; Sigma, St Louis, MO, USA) and rechromatographed on the same column, resulting in two fractions: the unbound fraction and the bound fraction (fraction III).

The glycopeptides originated from microsomal β-glucuronidase were separated into seven fractions as follows: I, the fraction that passed through Con A but was retarded on RCA I; II, the fraction that passed through Con A but was bound to RCA I; III, the fraction that was bound weakly to Con A, passed through WGA and bound to LCA; IV, the fraction that was bound weakly to Con A and passed through all of WGA, LCA and UEA; V, the fraction that was bound weakly to Con A, passed through WGA and LCA, and was bound to UEA; VI, the fraction that was bound strongly to Con A and passed through LCA and WGA; VII, the fraction that was bound strongly to Con A, passed through LCA and bound to WGA (Fig. 2).

The glycopeptides originated from lysosomal β-glucuronidase were separated into six fractions as follows: I, the fraction that passed through Con A but was retarded on RCA I; II, the fraction that passed through Con A but was bound to RCA I; III, the fraction that was bound weakly to Con A and bound to WGA; IV, the fraction that was bound weakly to Con A, passed through WGA, and was bound to LCA; V, the fraction that was bound weakly to Con A and passed through all of WGA, LCA and UEA; C, the fraction that was bound strongly to Con A (Fig. 3).

According to the distribution of the radioactivity in various fractions, and assuming that all glycopeptides were acetylated to the same specific radioactivity, the relative amounts of different glycan structure types are shown in the brackets in Figs. 2 and 3.

## 4. Discussion

Sequential affinity chromatography using immobilised lectins allowed us to better understand and complete previous data concerning rat liver microsomal and lysosomal β-glucuronidase glycan structures.

Based on the results of the present study and the lectin binding studies performed by others [19,26–29] (Table 1), as well as on knowledge of rat liver protein glycosylation [14,30–32], it may be concluded that fractions I and II, obtained from microsomal and lysosomal enzymes using the RCA I-Sepharose column, represent complex tri- and/or tetraantennary type glycans with different amounts of terminal galactose residues (one or two galactose residues in the case of retarded fraction and three or more galactose residues in the case of tightly bound fractions [19,23,33,34]. The absence of *O*-glycan, GalNAcα→Ser/Thr, in both enzymes was shown by the lack of binding of Con A-Sepharose unbound fraction (A′) to immobilised soybean agglutinin. Additionally, our observation is confirmed by the previous reports, showing that *O*-glycosidically-linked oligosaccharides from rat liver are mainly Galβ1→3GalNAcNα→Ser/Thr and its monosialosyl and disialosyl derivatives [30,35]. None of glycopeptides obtained from microsomal or lysosomal β-glucuronidase bound to the $E_4$-PHA-agarose column. This indicates that these enzymes do not possess bisected complex bi- and triantennary type glycans with galactose residue on the Manα1→6 arm [36]. Additionally, according to previous report, rat liver glycoproteins do not have bisected *N*-linked sugar chains. The result obtained may be also supported by the study reporting low level of GnT-III (*N*-acetylglucosaminyltransferase III, the enzyme responsible for synthesis of bisected *N*-linked oligosaccharides) in all mammalian hepatocytes [37]. The fractions obtained from both isoenzymes, which pass unbound to $E_4$-PHA-agarose column (fractions 0),

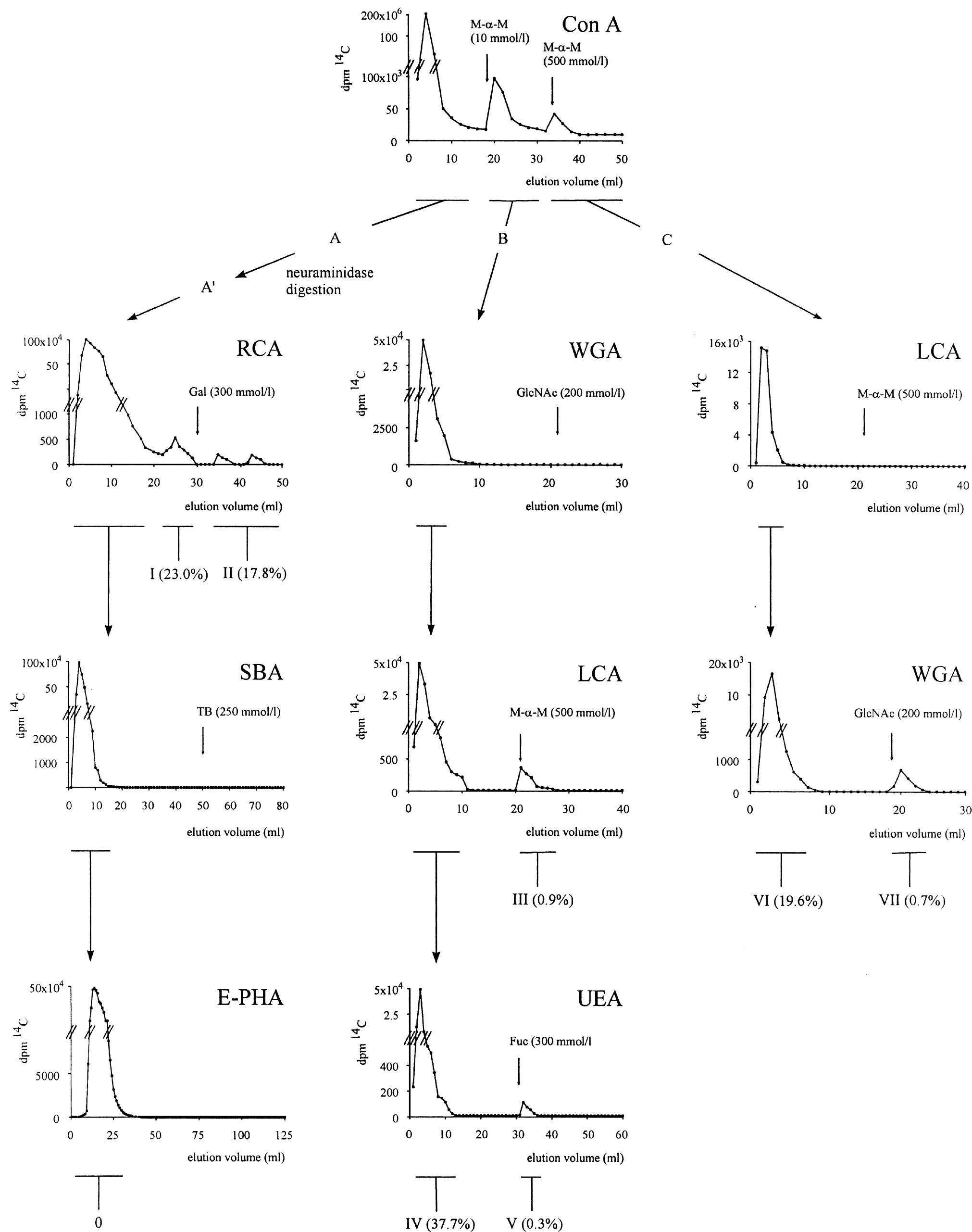


Fig. 2. Separation of $N$-[$^{14}$C]acetylated microsomal β-glucuronidase glycopeptides into seven fractions by serial lectin affinity chromatography. Fractions A′ (fraction A digested with neuraminidase), B and C obtained on the Con A column were subsequently applied to the RCA, WGA and LCA columns, respectively. The unbound fractions on the RCA, WGA and LCA columns were further applied to the SBA, LCA and WGA columns, respectively. The unbound fractions on the SBA and LCA columns were further applied to the $E_4$-PHA and UEA columns, respectively. The arrows running between different chromatographic profiles indicate sequence of steps. The arrows within the graphs indicate the starting points of elution. The horizontal bars indicate fractions, which were collected and subsequently applied to a next lectin affinity column. Relative amounts of different glycan structure types for microsomal β-glucuronidase are given in the brackets. These values were calculated on the assumption that all glycopeptides have the same specific radioactivity. α-MM, α-methyl-D-mannoside; Fuc, L-fucose; Gal, galactose; GlcNAc, $N$-acetylglucosamine; TB, sodium tetraborate.

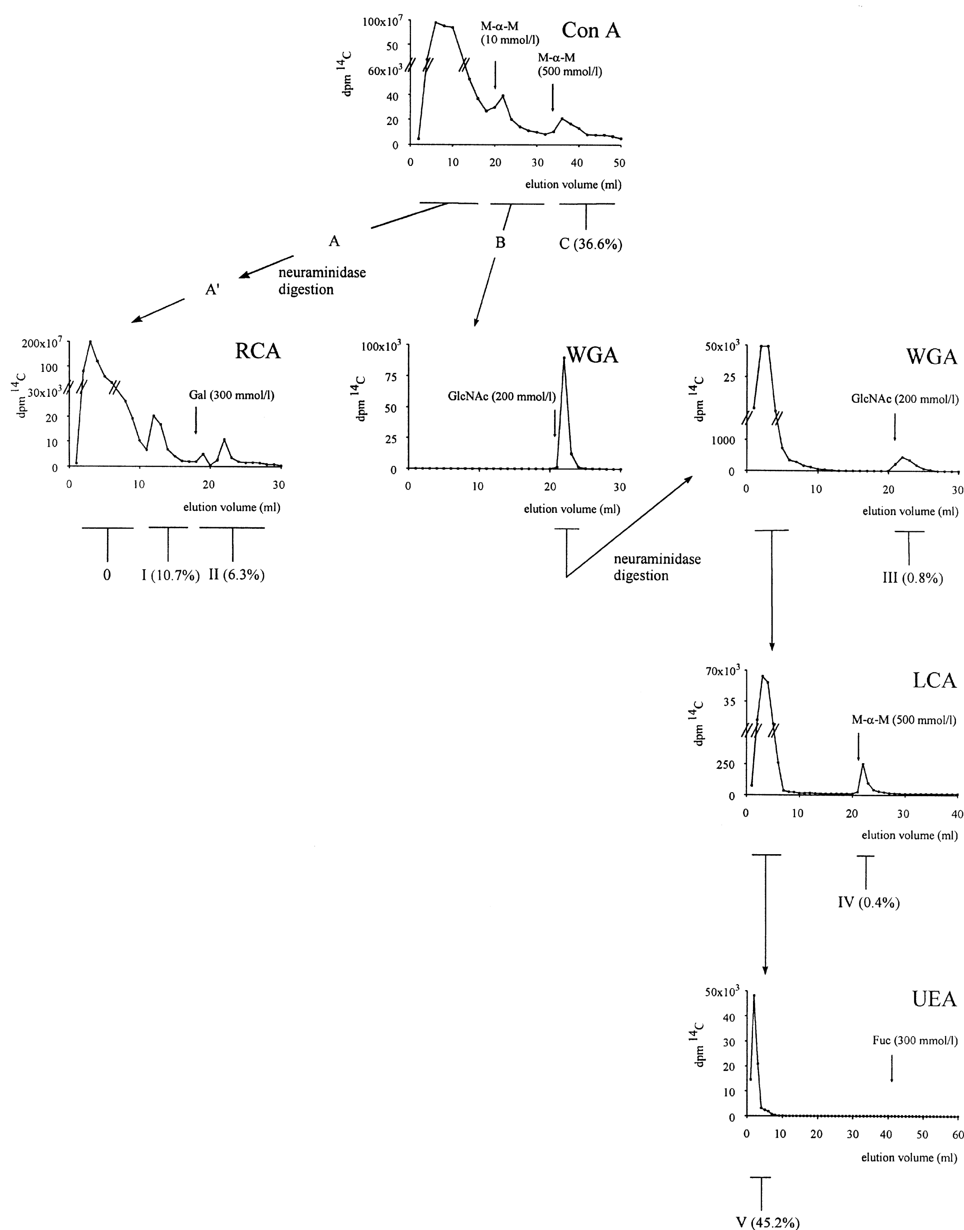


Fig. 3. Separation of $N$-[$^{14}$C]acetylated lysosomal β-glucuronidase glycopeptides into five fractions by serial lectin affinity chromatography. Fractions A′ (fraction A digested with neuraminidase), B and C obtained on the Con A column were subsequently applied to the RCA, WGA and LCA columns, respectively. The bound fraction on the WGA column was digested with neuraminidase and rechromatographed on the WGA column to check if sialic acid residues were involved in the interaction with the lectin. The unbound fraction on the WGA column was further applied to the LCA column, and the unbound fraction on the LCA column was subsequently applied to the UEA column. The arrows running between different chromatographic profiles indicate sequence of steps. The arrows within the graphs indicate the starting points of elution. The horizontal bars indicate fractions, which were collected and subsequently applied to a next lectin affinity column. The scheme shows only these chromatographic steps of fractionation of lysosomal glycopeptides, which were different in comparison with previously reported results [14]. Relative amounts of different glycan structure types for lysosomal β-glucuronidase are given in the brackets. These values were calculated on the assumption that all glycopeptides have the same specific radioactivity. α-MM, α-methyl-D-mannoside; Fuc, L-fucose; Gal, galactose; GlcNAc, $N$-acetylglucosamine.

might include mainly acetylated amino acids and peptides [14]. Moreover, one can speculate that these fractions may also contain complex tri- and tetra-antennary type glycans lacking their terminal galactose residues, or complex biantennary type glycans with fucose$\alpha$1$\rightarrow$3 residues bound to outer *N*-acetylglucosamine [30] or some *O*-glycosidically-linked oligosaccharides [30,38]. Also, there is a possibility that fractions 0 may contain bisected hybrid type glycans, because *N*-glycosidically-linked oligosaccharides of total purified glycopeptides from rat liver, which do not bind to Con A, have been shown to possess 6,4,3-tri-*O*-substituted mannose residues [30]. The total radioactivity of lysosomal fraction B was bound to the WGA-agarose column. After the desialylation, only 0.8% of this fraction was still able to bind to WGA. It suggests that lysosomal fraction B consists of complex biantennary type glycans with clustered sialic acid residues [25,39,40], while fraction III possesses only non-complete complex biantennary type glycans with terminal GlcNAc residues [31]. The presence of poly-*N*-acetyllactosamine chains and bisected hybrid type glycans should be excluded, because these residues were not detected in the fraction containing total oligosaccharides isolated from rat liver Golgi apparatus and bound to Con A [19,30,31,41].

The absence of hybrid type oligosaccharides in rat liver could be explained by a low level of GnT-III expression in this tissue [31]. Together with $\alpha$-mannosidase II (the enzyme, which removes one $\alpha$1$\rightarrow$3 and one $\alpha$1$\rightarrow$6 linked mannose residue from Man$\alpha$1$\rightarrow$6 arm to yield $GlcNAcMan_3GlcNAc_2$), GnT-III is one of the most important enzymes involved in the regulation of hybrid and/or a complex type oligosaccharides synthesis.

In contrast to lysosomal enzyme, the total radioactivity of microsomal fraction B did not bind to WGA-agarose. This means that complex biantennary type glycans present in this fraction possess terminal galactose residues unsubstituted with sialic acid. The presence of fraction III for microsomal and fraction IV for lysosomal $\beta$-glucuronidase suggests that both enzymes have complex biantennary type glycans with fucose $\alpha$1$\rightarrow$6 linked to innermost GlcNAc residue [42]. The presence of fraction V which consists of microsomal glycopeptides suggests that complex biantennary type glycans contain Fuc$\alpha$1$\rightarrow$2 linked to outer Gal$\beta$1$\rightarrow$4 residue [43]. The presence of small number of fucosylated residues may result from a low level of expression of fucosyltransferases in rat liver [44]. Both fractions IV and V (for microsomal and lysosomal $\beta$-glucuronidase, respectively) possess only unfucosylated complex biantennary type glycans, because Fuc$\alpha$1$\rightarrow$4 residue, linked to outer GlcNAc, was not found in rat liver [30]. Moreover, the presence of some truncated hybrid type structures could not be excluded [31]. Fraction VII containing 0.7% of the total microsomal $\beta$-glucuronidase glycopeptides represents non-complete complex biantennary type glycans with terminal GlcNAc residues [31]. The presence of bisected hybrid type glycans should be excluded, since these kinds of oligosaccharides were not found in rat liver oligosaccharides fraction bound to Con A-Sepharose [19,30,31,41]. In the case of both isoenzymes, fractions that were first strongly bound to Con A, and then passed unbound to LCA, and finally bound to WGA, contain high mannose and/or non-bisected hybrid type glycans.

Mizouchi et al. [12] isolated and analysed *N*-glycan structures from rat liver microsomal and lysosomal $\beta$-glucuronidases. They reported only the presence of high mannose type oligosaccharides. Oligosaccharides were released by two different methods: (1) enzymatic (endo-$\beta$-*N*-acetyl-glucosaminidase treatment (endoH)), and (2) chemical (hydrazinolysis). However, endoH is not able to release complex type glycans from glycopeptides [45]. Additionally, the authors did not confirm if deglycosylation using endoH was performed to completeness. Moreover, the conditions of hydrazinolysis (100°C for 10 h) were harsh and might cause degradation of some carbohydrates [46]. Kozutsumi et al. [13] demonstrated that small part of lysosomal $\beta$-glucuronidase from rat liver possesses *N*-acetyllactosamine type oligosaccharides while the majority of this enzyme seems to have only oligomannoside-type glycans. To analyse carbohydrate structures, Kozutsumi and co-workers used lectin-affinity chromatography on the RCA I-agarose and MBP-Sepharose column (that is specific for glycoproteins containing *N*-acetyllactosamine type or oligomannoside type glycans). The authors concluded that only 3% of $\beta$-glucuronidase activity was interacting with the RCA I-agarose column. How-

ever, the authors did not remove sialic acid and did not denature β-glucuronidase before chromatography on lectin column. Therefore, not all oligosaccharide residues were exposed for the binding with this lectin.

In our previous study on rat liver lysosomal β-glucuronidase [14], glycopeptides obtained by Pronase digestion were analysed by sequential lectin affinity chromatography. To analyse neuraminidase treated and not bound to Con A-Sepharose fraction, we have used the RCA I-agarose column. However, we did not detect any radioactivity bound to this lectin column. Pronase digestion is one of the most frequently used approaches to convert glycoproteins into glycopeptides, small-sized peptides and amino acids. For some reason [47], Pronase cannot efficiently cleave the peptide regions between the glycosylated residues, resulting in the production of large size glycopeptides containing multiple oligosaccharide chains. Perhaps that is why we could not detect any retarded and bound fractions on RCA I-agarose.

None of the studies performed so far revealed the presence of complex tri- and/or tetraantennary type glycans on rat liver microsomal and lysosomal β-glucuronidase molecules. On the other hand, our results, concerning the presence of complex type glycans, are coincident with the chemical analysis of microsomal and lysosomal β-glucuronidase carbohydrates [9,11], indicating that both enzymes contain mannose, galactose, fucose, glucosamine and sialic acid residues.

Neuraminidase treatment of fraction A allowed removal of sialic acid and, consequently, exposure of galactose residues able to interact with RCA I-Sepharose. The presence of sialic acid bound to galactose residues abolishes the interaction of glycopeptides with RCA I-Sepharose, SBA-agarose and $E_4$-PHA-agarose [22–24] but does not abolish the interaction with Con A-Sepharose [26]. Microsomal β-glucuronidase glycopeptides present in fraction B did not bind to the WGA-agarose column, therefore they did not need neuraminidase treatment. In contrast, lysosomal β-glucuronidase glycopeptides present in fraction B were bound to WGA-agarose. Thus, they required neuraminidase treatment. This novel approach enabled further analysis and confirmed that lysosomal enzyme contains sialylated complex biantennary type glycans.

Lectin column chromatography is a useful and unique technique for fractionation of oligosaccharides and glycopeptides, which is based on carbohydrate binding specificity of lectins. Although this method allows fractionation of glycopeptides into type and amount of glycans present in the mixture, it does not provide full information about their structures and masses. More detailed studies could be done to elucidate glycan structures using other methods such as HPLC and mass spectrometry.

The presence of triantennary complex type glycan in microsomal β-glucuronidase was confirmed using exoglycosidases digestion and HPLC analysis (RAAM) (data not published).

## 5. Conclusions

We were able to show that rat liver microsomal β-glucuronidase possesses: 40.8% complex tri- and/or tetraantennary type glycans (17.8% reveals three or more terminal galactose residues and 23% reveals one or two terminal galactose residues), 38.9% non-sialylated complex biantennary type glycans and 19.6% high mannose and/or non-bisected hybrid type glycans. Lysosomal rat liver β-glucuronidase possesses:17.0% complex tri- and/or tetraantennary type glycans (6.3% reveal three or more terminal galactose residues and 10.7% reveal one or two galactose residues), 46.4% sialylated complex biantennary type glycans and 36.6% high mannose and/or non-bisected hybrid type glycans.

## Acknowledgements

The authors wish to thank Maria Zygmunt for her excellent technical assistance. This study was supported by grants from the Jagiellonian University DS/IZ/FZ/99 and BW/34/IZ/2000.

## References

[1] C. De Duve, B.C. Pressman, R. Gianetto, R. Wattiaux, F. Appelmans, Biochem. J. 60 (1955) 604.
[2] S. Medda, R.M. Chemellis, J.L. Martin, L.R. Pohl, R.T. Swank, J. Biol. Chem. 264 (1989) 15824.

[3] P.D. Stahl, O. Touster, J. Biol. Chem. 246 (1971) 5398.
[4] L. Zhen, M.E. Rusiniak, T. Swank, J. Biol. Chem. 270 (1995) 11912.
[5] S. Medda, A.M. Stevens, R.T. Swank, Cell 50 (1987) 301.
[6] H. Tsuji, N. Hattori, T. Yamamoto, K. Kato, J. Biochem. 82 (1977) 619.
[7] J.W. Owens, P. Stahl, Biochim. Biophys. Acta 438 (1976) 474.
[8] M. Himeno, Y. Nishimura, H. Tsuji, K. Kato, Eur. J. Biochem. 70 (1976) 349.
[9] D.R.P. Tulsiani, H. Six, O. Touster, Proc. Natl. Acad. Sci. USA 75 (1978) 3080.
[10] J.M. Shipley, J.H. Grubb, W.S. Sly, J. Biol. Chem. 268 (1993) 12193.
[11] M. Himeno, Y. Nishimura, K. Takahashi, K. Kato, J. Biochem. 83 (1978) 511.
[12] T. Mizouchi, Y. Nishimura, K. Kato, A. Kobata, Arch. Biochem. Biophys. 209 (1981) 298.
[13] Y. Kozutsumi, T. Kawasaki, I. Yamashina, J. Biochem. 100 (1986) 505.
[14] B. Wójczyk, D. Hoja, A. Lityńska, Glycoconj. J. 10 (1993) 175.
[15] J.W. Owens, K.L. Gammon, P.D. Stahl, Arch. Biochem. Biophys. 166 (1975) 258.
[16] M.L. Watson, J. Biophys. Biochem. Cytol. 4 (1958) 475.
[17] E.S. Reynolds, J. Cell Biol. 17 (1963) 208.
[18] Pharmacia Fine Chemicals, in: Polyacrylamide Gel Electrophoresis, Rahms i Lund, Uppsala, Sweden, 1980, p. 69.
[19] M. Bierhuizen, M. Hansson, P. Odin, H. Debray, B. Obrink, W. van Dijk, Glycoconj. J. 6 (1989) 195.
[20] B. Wójczyk, D. Hoja, A. Lityńska, Glycoconj. J. 8 (1991) 340.
[21] M.M. Bradford, Anal. Biochem. 272 (1976) 248.
[22] H. Debray, D. Decout, G. Strecker, G. Spik, J. Montreuil, Eur. J. Biochem. 117 (1981) 41.
[23] J.U. Baenziger, D. Fiete, J. Biol. Chem. 254 (1979) 9795.
[24] E.D. Green, J.U. Baenzinger, J. Biol. Chem. 262 (1987) 12018.
[25] M. Monsigny, A.C. Roche, C. Sene, R. Maget-Dana, F. Delmotte, Eur. J. Biochem. 104 (1980) 147.
[26] R.K. Merkle, R.D. Cummings, Methods Enzymol. 138 (1987) 232.
[27] J.G. Beeley, in: R.H. Burdon, P.H. van Knippenberg (Eds.), Glycoprotein and Proteoglycan Techniques, Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 16, Elsevier, Amsterdam, 1985, p. 301.
[28] A. Kobata, K. Yamashita, in: M. Fukuda, A. Kobata (Eds.), Glycobiology. A Practical Approach, Oxford University Press, Oxford, 1993, p. 103, Chapter 3B.
[29] T. Endo, J. Chromatogr. A 720 (1996) 251.
[30] T. Kurius, J. Finne, Eur. J. Biochem. 78 (1977) 369.
[31] B.K. Hayes, A. Varki, J. Biol. Chem. 268 (1993) 16155.
[32] V.N. Subramaniam, A.R. bin Mohd. Yusoff, S.H. Wong, G.B. Lim, M. Chew, W. Hong, J. Biol. Chem. 267 (1992) 12016.
[33] H. Debray, A. Pierce-Cretel, G. Spik, J. Montreuil, in: T.C. Bøg-Hansen, G.A. Spengler (Eds.), Lectins, Vol. III, Walter de Gruyter, Berlin, 1983, p. 335.
[34] Y. Shinohara, H. Sota, F. Kim, M. Shimizu, M. Goth, M. Tosu, Y. Hasegawa, J. Biochem. 117 (1995) 1076.
[35] J. Finne, T. Krusius, FEBS Lett. 66 (1976) 94.
[36] K. Yamashita, A. Hitoi, A. Kobata, J. Biol. Chem. 258 (1983) 14753.
[37] A. Kobata, Eur. J. Biochem. 209 (1992) 483.
[38] B.K. Hayes, A. Varki, J. Biol. Chem. 268 (1993) 16170.
[39] K. Yamamoto, T. Tsuji, I. Matsumoto, T. Osawa, Biochemistry 20 (1981) 5894.
[40] R.J. Ivantt, J.W. Reeder, G.F. Clark, Biochim. Biophys. Acta 883 (1986) 253.
[41] S. Oda-Tamai, S. Kato, N. Acamatsu, Biochem. J. 280 (1991) 179.
[42] K. Kornfeld, M.L. Reitman, R. Kornfeld, J. Biol. Chem. 256 (1981) 6633.
[43] T. Matsui, K. Titani, T. Mizouchi, J. Biol. Chem. 267 (1992) 8723.
[44] S. Hartel-Schenk, N. Minnifield, W. Reutter, C. Hanski, C. Bauer, D.J. Morré, Biochim. Biophys. Acta 1115 (1991) 108.
[45] A. Kobata, T. Endo, in: M. Fukuda, A. Kobata (Eds.), Glycobiology. A Practical Approach, Oxford University Press, Oxford, 1993, p. 79, Chapter 3A.
[46] S. Takasaki, T. Mizouchi, A. Kobata, Methods Enzymol. 83 (1982) 263.
[47] R.D. Cummings, in: M. Fukuda, A. Kobata (Eds.), Glycobiology. A Practical Approach, Oxford University Press, Oxford, 1993, p. 253, Chapter 5.