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Purification of β-glucuronidase and structural assessment of the carbohydrate chains by lectin affinity immunoelectrophoresis

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The purification of rat liver β-glucuronidase from a lysosomal fraction by methods including affinity chromatography, chromatofocusing and preparative PAGE steps is described. Molecular weights of 300 000 and 150 000 were estimated by two dimensional gradient PAGE/immunoelectrophoresis of the lysosomal extract. Isoelectrofocusing in agarose gel followed by immunoelectrophoresis in the second dimension revealed the presence of at least five maxima in the range pH 4.3–7.4.

The structural assessment of the carbohydrate chains of lysosomal and microsomal β-glucuronidase was performed by lectin affinity immunoelectrophoresis. Reaction with Concanavalin A indicated the presence of bi-antennary complex, oligomannosidic and hybrid type structures, whereas the absence of tri- and tetra-antennary complex type structures was deduced from the lack of interaction with phytohemagglutinin-L. The reaction with *Lens culinaris agglutinin*, *Pisum sativum* agglutinin and *Lotus tetragonolobus* lectin revealed that part of the glycans contained a fucose α(1-6)-linked to the *N*-acetylglucosamine attached to asparagine. The presence of terminal β(1-4)-galactose residues was detected with *Ricinus communis* agglutinin I.

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

β-Glucuronidase (EC 3.2.1.31) is a glycoprotein acid hydrolase like most other soluble lysosomal enzymes, widely distributed in mammalian tissues. Moreover, β-glucuronidase is associated with both the lysosomal and microsomal fraction [1]. Since the carbohydrate moiety of the enzyme determines its subcellular localization [2–4] we decided to investigate the carbohydrate chains of this enzyme. In the present study we report a purification procedure as well as a qualitative assessment of the carbohydrate structures of β-glucuronidase from a rat liver lysosomal fraction. This was performed by different chromatographic methods and by lectin affinity immunoelectrophoresis.

Materials and methods

Materials

The following materials were obtained from the suppliers indicated: Sephadex, Sepharose and derivatives, Polybuffer 74, Pharmalyte 3-10, Low and High Molecular Weight Calibration Kit, Concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), Wheat germ agglutinin (WGA), Soybean agglutinin (SBA), and *Helix pomatia* agglutinin (HPA) were from Pharmacia LKB Biotechnology, Uppsala, Sweden; agarose and acrylamide were from Serva-Feinbiochemica, Heidelberg, Germany; phenolphthalein glucuronic acid, *Phytolacca americana* agglutinin (PAL) and Phytoheam-agglutinin P (PHA) were from Sigma Chemical Co., St Louis, MO, USA; *Dolichos biflorus* agglutinin (DBA) was from Polfa, Poland; *Lotus tetragonolobus* agglutinin (LTL) and *Ricinus communis* agglutinin (RCA_I) were kindly supplied by Dr H. Debray (Lille, France); *Moluccella laevis* agglutinin (MLA) and *Erythrina corallodendron* agglutinin (ECA) were gifts from Professor N. Sharon (Rehovot, Israel). Erythro-agglutinin (PHA-E), Leukoagglutinin (PHA-L) from *Phaseolus vulgaris* and *Pisum sativum* agglutinin (PSA) were prepared in our laboratory as already described [5, 6]. All other reagents were of the highest grade available.

Isolation of rat liver lysosomal fraction

Lysosomal preparation was carried out by a modification of the procedure described by de Duve *et al.* [1]. All subsequent steps were carried out at 4°C. Livers from Wistar male rats (total, 1000–1250 g) were removed and homogenized in 0.25 M sucrose, 1 mM EDTA, pH 7.4 (5 ml per g tissue) using a motor-driven Potter–Elvehjem homogenizer at 600 rev/min. The homogenate was centrifuged at

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400 × *g* for 10 min. The pellet obtained was homogenized and centrifuged once again. The combined postnuclear fraction was centrifuged at 4000 × *g* for 15 min. An enriched lysosomal pellet was obtained by centrifugation of post-4000 × *g* supernatant at 25000 × *g* for 30 min (VAC-602, 6 × 85 ml rotor, VEB Janetzky). The resulting pellet was resuspended in 20 mM Tris-HCl buffer, 0.2 M NaCl, 0.2% Triton X-100, pH 7.4 (1 ml per g fresh liver), frozen and thawed twice, and centrifuged at 105000 × *g* for 90 min. The supernatant was collected, dialysed and used as a crude lysosomal extract.

Enzyme assay

β-Glucuronidase was assayed [7], the method being slightly modified. 30 µl enzyme solution was incubated with 30 µl 0.3 M acetate buffer, pH 4.5, containing 6 mM phenolphthalein glucuronic acid for an appropriate time at 37°C. The reaction was stopped by the addition of 0.8 ml 0.5 M glycine + NaOH buffer, pH 10.4, and the absorbance at 555 nm was measured. One unit of the enzyme hydrolyses 1 nmol of substrate per second (1 nkatal).

Protein determination

Protein was determined by a dye-binding assay method [8] using bovine serum albumin as standard.

Purification of β-glucuronidase from lysosomal extract

1. *Con A-Sepharose chromatography.* Crude lysosomal extract was applied to a column (2.6 × 18 cm) of Con A-Sepharose 4B equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 0.2 M NaCl, 1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$ [9], and unbound material was washed from the column with the same buffer. Adsorbed proteins were eluted with starting buffer containing 0.5 M α-methyl-D-glucoside, monitoring the effluent at 280 nm and collecting 16 ml fractions at a flow rate of 60 ml h^{-1}. The fractions containing enzymatic activity were pooled and dialysed against 10 mM Tris-CH_3COOH buffer, pH 6.0.

2. *Blue Sepharose chromatography.* The material from the previous step was applied to a column (2.6 × 18 cm) of Blue Sepharose Cl 6B equilibrated with 10 mM Tris-CH_3COOH buffer, pH 6.0 [10]. After thorough washing with starting buffer to remove unbound material, inert protein was removed by 10 mM Tris-HCl buffer, pH 7.4, and β-glucuronidase was eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl. The flow rate was 40 ml h^{-1} and 12 ml fractions were collected. The pooled active fractions were dialysed against 25 mM Tris-HCl, pH 7.2.

3. *Chromatofocusing.* The Polybuffer exchanger PBE 94 was poured into a column (1 × 35 cm) and equilibrated with 600 ml of 25 mM Tris-HCl buffer, pH 7.2, at a flow rate of 35 ml h^{-1}. The β-glucuronidase preparation was applied and washed into the column with 6 ml buffer. The column was developed with 320 ml of Polybuffer (35.5 ml of concentrated Polybuffer adjusted to pH 4.0 with 0.5 M HCl and diluted to 320 ml), monitoring the pH of each 2.5 ml fraction. The elution profile is shown in Fig. 1. Fractions containing β-glucuronidase activity were pooled and dialysed against 10 mM Tris-HCl buffer, pH 6.4.

4. *DE-52 ion exchange chromatography.* A sample of β-glucuronidase was applied to a column (2.6 × 10 cm) of DEAE cellulose DE-52 equilibrated with 10 mM Tris-HCl buffer, pH 6.4, and unbound material was washed from the column with the same buffer. Adsorbed enzyme was eluted with a linear gradient of NaCl (100 ml H_2O + 100 ml 0.5 M NaCl) and a flow rate of 45 ml h^{-1}, collecting 2 ml fractions. Fractions containing enzymatic activity were pooled, concentrated and dialysed against 10 mM Tris-HCl buffer, pH 6.4, containing 0.15M NaCl.

5. *Gel filtration.* 2.5 ml was applied to a column (1.3 × 52 cm) of Sepharose Cl-6B equilibrated with 10 mM Tris-HCl buffer pH 6.4 containing 0.15 M NaCl. Fractions (1.5 ml) were collected with a flow rate of 30 ml h^{-1}. β-Glucuronidase-containing fractions were pooled, concentrated and dialysed against 5 mM Tris-glycine buffer, pH 8.6.

6. *Preparative electrophoresis.* An 8% separating and 3.5% stacking polyacrylamide gel was used in the electrophoresis for final purification of the β-glucuronidase with 5 mM Tris-glycine, pH 8.6, as gel buffer and 5 mM Tris-glycine-HCl, pH 8.3, as electrophoretic buffer. Preparative electrophoresis was performed in the apparatus described elsewhere [11]. The concentrated (0.7 ml) β-glucuronidase-containing sample with the addition of bromophenol blue, was applied to the top of the gel (diameter 3 cm, length of the stacking gel 0.5 cm and separating gel 1.2 cm). Electrophoresis was carried out at 200 V with the anode at the gel bottom. As soon as the dye reached the bottom of the gel, the collecting chamber was emptied every 3 min. Fractions (2 ml each) possessing the highest enzyme activity were tested for purity by polyacrylamide gel electrophoresis in rods. The separation profile is shown in Fig. 2. The final preparation was dialysed against distilled water, prior to lyophilization. In each case concentration was performed by ultrafiltration on a Diaflo UM 10 membrane.

Antibody production

Rabbits were immunized with purified β-glucuronidase at approximately three week intervals. The enzyme (82 µg, 500 µl) was emulsified with complete Freund's adjuvant (1.5 ml) and injected intradermally into multiple sites along the rabbit's back. Two booster intramuscular injections contained similar amounts of antigen emulsified with incomplete Freund's adjuvant. The presence of antibody was monitored by double immunodiffusion in 1.5% agarose gel as described by Ouchterlony [12]. After nine weeks the blood was collected by catheterization of the carotid. The blood was allowed to clot at 37°C for 1 h and the clot was then allowed to contract at 4°C for 12 h. The serum was

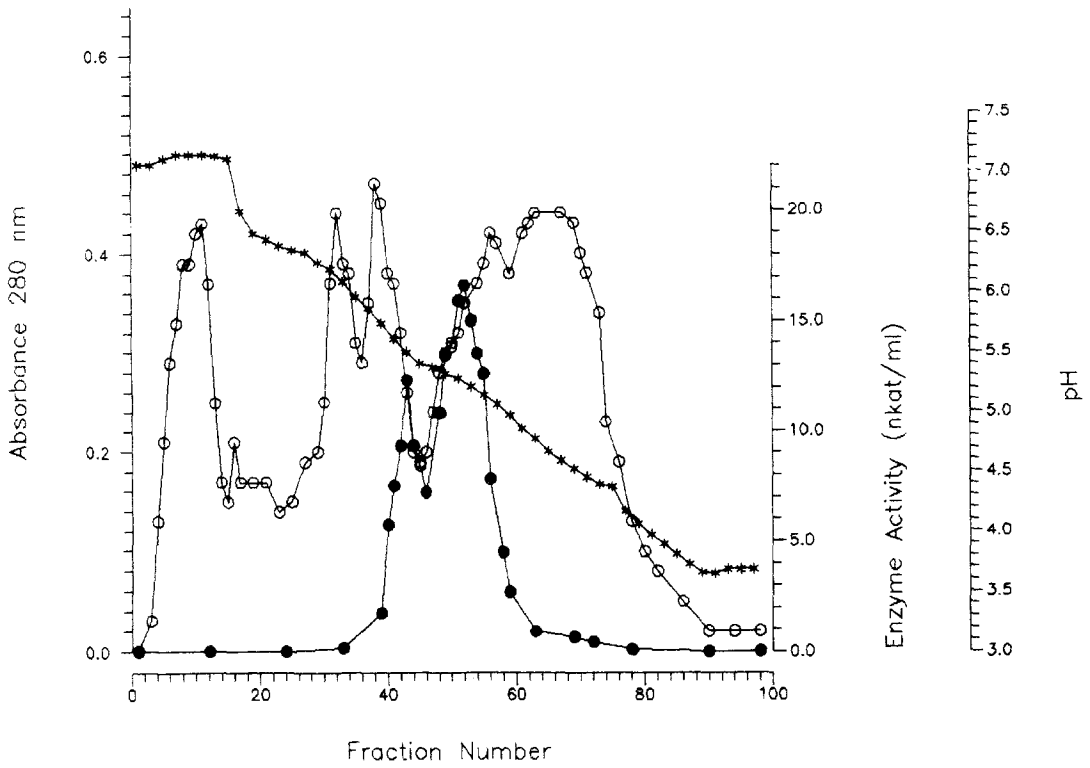


Figure 1. Chromatofocusing of β-glucuronidase from rat liver lysosomal fraction. The column (1 cm × 35 cm) of PBE 94 gel was equilibrated with 25 mM Tris-HCl buffer, pH 7.2, and developed with Polybuffer 74, pH 4.0. The flow rate was 35 ml h^{-1} and the fraction volume was 2.5 ml. Protein concentration (○), enzyme activity (●), and pH (*) of the eluate were monitored.

collected after centrifugation and stored at 4°C in the presence of 0.015 M NaN_3. The serum obtained was used for two dimensional and rocket immunoelectrophoresis.

Electrophoretic techniques

Polyacrylamide gel electrophoresis was performed as described in [13] in 7.5% gel rods for 1–1.5 h at 1–2 mA per tube. In all instances protein bands were visualized by staining with Coomassie Brilliant Blue R-250 [14].

Rocket immunoelectrophoresis was performed [15] in 1% agarose gel in 0.07 M Tris-barbital buffer, pH 8.9, at 2 V cm^{-1} for 19 h. Purified β-glucuronidase from the lysosomal fraction (for details see the Materials and methods section) and from the calcium precipitated microsomal fraction [16] were applied to sample wells together with one of the following lectins: Con A, LCA, PSA, WGA, RCA_I, LTL, PHA-P, PHA-L, PHA-E, SBA, HPA, DBA, PAL, MLA, ECA, CAA (15, 30, 50 μg lectin per well) or without lectin, as a control. Each lectin was dissolved in 1 mM Tris-barbital buffer, pH 6.8, containing 1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$. β-Glucuronidase immunoprecipitates were detected as rockets by rabbit antiglucuronidase serum incorporated into the upper gel (0.9 μl cm^{-2}). The distance between the application wells and antibody gel was 8 mm. Plates were pressed, washed and stained with Coomassie Brilliant Blue R-250. The area under each rocket was copied on tracing paper, cut out and weighed. Interaction of β-glucuronidase with each lectin was expressed as the percentage of each rocket weight in comparison to the weight of the control rocket.

Two dimensional immunoelectrophoresis was performed as described [17]. In the first dimension the sample was separated in 1% agarose gel (control separation) in 0.07 M Tris-barbital buffer, pH 8.8, at 10 V cm^{-1} of gel until the albumin spot migrated 5 cm. Alternatively, the first separation was performed (i) by polyacrylamide gradient gel electrophoresis, (ii) by isoelectric focusing in 1% agarose gel, or (iii) in 1% agarose gel containing different lectins. In the second dimension, electrophoresis was performed in 1% agarose antibody containing gel at 2 V cm^{-1} for 18 h. Plates were pressed, washed and stained with Coomassie Brilliant Blue R-250.

(i) Polyacrylamide gradient gel electrophoresis was performed [14] in slabs (70 × 75 × 1 mm). The acrylamide gel concentration was between 4 and 30% and electrophoresis was carried out at 90 V for 16 h. Gels were calibrated with a high molecular weight calibration kit consisting of thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

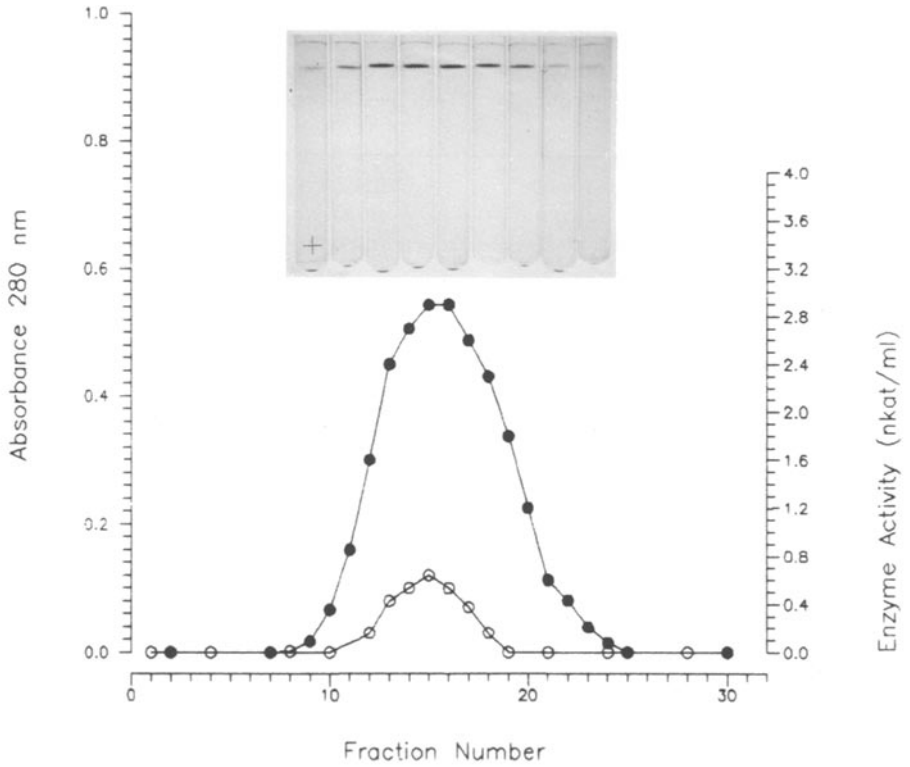


Figure 2. Preparative polyacrylamide gel electrophoresis of β-glucuronidase from a rat liver lysosomal fraction. Electrophoresis was carried out in 8% separating gel (1.2 cm × 3 cm) and 3.5% stacking gel (0.5 cm × 3 cm) at 200 V using 5 mM Tris-glycine-HCl buffer, pH 8.3. Fractions of 2 ml were collected every 3 min. Protein concentration (○) and enzyme activity (●) in every fraction were monitored. Fractions possessing the highest enzyme activity were tested for purity by electrophoresis in 7.5% polyacrylamide gel rods.

(ii) Isoelectrofocusing was performed as described in [18] in 1% agarose (IEF agarose) containing 12% sorbitol. A gradient of pH 3–10 was established by a concentration of 6% Pharmalyte. 1 M NaOH and 0.05 M H_2SO_4 were used as cathodal and anodal solutions. Electrofocusing was carried out at 10°C for 2 h with a maximum voltage of 1500 V and constant power of 15 W. Approximately 8 μl samples were applied in the middle of the gel, directly on the surface. The gel was calibrated with an isoelectric focusing calibrating kit consisting of amyloglucosidase (pI 3.5), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), myoglobin (pI 6.85; 7.35), lentil lectin (pI 8.15; 8.45; 8.65), trypsinogen (pI 9.3)

(iii) Lectin affinity electrophoresis was performed according to the technique developed by Bøg-Hansen [19]. Agarose gel (1%) contained one of the following lectins: Con A (300 μg cm^{-2}) and 50 μg cm^{-2} LCA, WGA, SBA, HPA, PAL, PHA, DBA and LTL. Intermediate gel containing either 20% α-D-methylmannoside, 20% D-galactose or, 20% *N*-acetyl-D-glucosamine or 10% L-fucose, depending on the specificity of the lectin used, was always included between first and second dimension gels.

Results and discussion

Enzyme purification

The seven step purification of the β-glucuronidase from the rat liver lysosomal fraction was monitored by enzyme activity and protein assays. The results are given in Table 1. The overall recovery of the purified enzyme was typically about 4% with a purification of approximately 200-fold, starting from crude lysosomal extract. Affinity chromatography on Con A-Sepharose was one of the most effective steps, since 95% of inert protein was removed during this step. β-Glucuronidase activity was not present at all in this inert protein (unbound fraction). However, the low purification (15-fold) is due to the loss of enzyme activity during elution with 0.5 M α-methyl-D-glucoside.

As a second step, affinity chromatography on Blue Sepharose was used according to the method of Ahmad *et al.* [10]. Under these conditions all enzyme activity was adsorbed to the column and eluted with 0.5 M NaCl. Chromatofocusing on PBE 94 gel resulted in separation of β-glucuronidase from many proteins (Fig. 1), including other lysosomal enzymes, e.g., arylsulphatases, β-*N*-acetyl-D-glucosaminidase and β-galactosidase. β-Glucuronidase was eluted in two peaks at pH 5.25 and 5.5. An ion exchange chromatography step enabled removal of 83% of inert

Table 1. Summary of the purification of β-glucuronidase. For full experimental details see the Materials and methods section. Purification stages: I, crude lysosomal extract; II, Con A-Sepharose 4B affinity chromatography; III, Blue Sepharose C1-6B chromatography; IV, Chromatofocusing; V, DEAE-cellulose DE-52 ion exchange chromatography; VI, Sepharose C1-6B gel filtration chromatography; VII, preparative electrophoresis.

Purification step	*Volume* (ml)	*Protein* (mg ml^{-1})	*Specific activity* (nkat mg^{-1})	*Yield* (%)	*Purification* (fold)
I	1400	0.6	5.8	100	1
II	158	0.13	89	38	15
III	35	0.075	321.6	18	56
IV	33	0.04	640	17.5	111
V	2.5	0.091	1253.1	5.9	218
VI	0.7	0.273	1132	4.5	197
VII	1	0.165	1200.1	4.1	209

protein, compared to the previous step. While gel filtration chromatography removed more inert protein, significant activity decrease was, however, observed. Final purification was achieved by preparative electrophoresis in polyacrylamide gel. Enzyme prepared by this procedure migrated on analytical polyacrylamide gel electrophoresis as a single protein band (Fig. 2) coincident with the enzyme activity band revealed by incubation of gel in the substrate solution. β-Glucuronidase has already been isolated from mouse liver homogenate [20] and rat liver lysosomal fraction [21] by multi-step, time-consuming procedures, consisting mostly of precipitation with ammonium sulphate or organic solvent, ion exchange chromatography and gel filtration. β-Glucuronidase from microsomal and lysosomal fractions of rat liver was also purified by antibody–Sepharose chromatography, but using antibodies raised against preputial gland enzyme [16, 22]. Our purification procedure resulted in a highly purified enzyme (although with a low yield) which was used to raise antibody. The monovalent rabbit antiserum obtained gave a single immunoprecipitate arch in two dimensional immunoelectrophoresis with crude lysosomal extract as antigen.

Molecular weight and isoelectric points

The molecular weight of the β-glucuronidase as determined under native conditions by polyacrylamide gradient (4–30%) gel electrophoresis was 300 000 and 150 000 using thyroglobulin, ferritin, catalase, lactate dehydrogenase and albumin as markers (Fig. 3). The peak corresponding to a mol. wt of 150 000 is very minor, indicating a low content of this form. For visualization of all β-glucuronidase forms recognized by antibodies, immunoelectrophoresis in the second dimension was employed. This increases the sensitivity considerably and allows molecular weight determination of a particular protein in crude extracts, without prior purification. A molecular weight of 76 000 for the β-glucuronidase subunit was estimated by polyacrylamide gradient gel electrophoresis in the presence of 0.2% sodium dodecyl sulfate (results not shown). It appears that the native form of β-glucuronidase is a tetramer, in agreement with data in the literature [20, 22, 23] but can also exist as a dimer (150 000) not mentioned before.

The pI of the β-glucuronidase as measured by chromatofocusing during the purification procedure were 5.25 and 5.5.

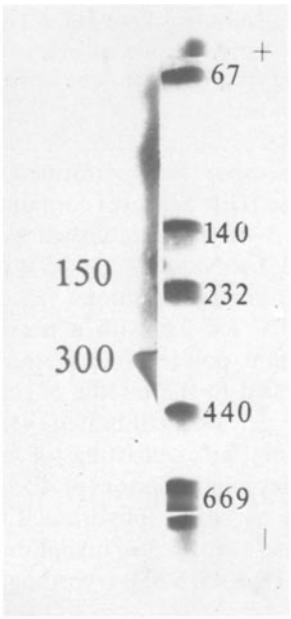


Figure 3. Two dimensional gradient (4–30%) PAGE-immunoelectrophoresis of crude lysosomal extract (10 µl). Gradient PAGE was carried out for 16 h at 90 V, immunoelectrophoresis was performed in 1% agarose containing 0.9 µl cm^{-2} rabbit antiglucuronidase serum for 18 h at 2 V cm^{-1}. First dimensional lane with molecular weight markers; thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa). Rockets of β-glucuronidase correspond to 300 kDa and 150 kDa. For full experimental details see the Materials and methods section.

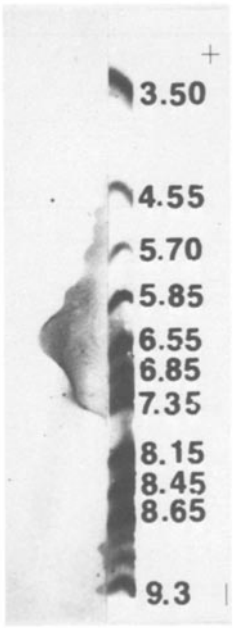


Figure 4. Two dimensional IEF-immunoelectrophoresis of crude lysosomal extract (8 µl). Separation in 1% agarose IEF containing 6% Pharmalyte pH 3–10 was carried out for 2 h at max. 1500 V, constant power 15 W. Immunoelectrophoresis was performed in 1% agarose containing 0.9 µl cm^{-2} rabbit antiglucuronidase serum for 18 h at 2 V cm^{-1}. First dimensional lane with markers; amyloglucosidase (pI 3.5), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), myoglobin (pI 6.85; 7.35), lentil lectin (pI 8.15; 8.45; 8.65) and trypsinogen (pI 9.3). β-Glucuronidase immunoprecipitated in the pH range 4.3–7.4.

However, enzyme activity was also found in fractions ranging from pH 5 to pH 5.7 (Fig. 1). The pI of the β-glucuronidase was also measured in crude lysosomal extract by isoelectrofocusing in 1% agarose gel in the pH range 3–10. In this case, the enzyme focused in the range of pH 4.3–7.4 with at least five pronounced maxima (main peak at about pH 6.0, Fig. 4). Again by the application of immunoelectrophoresis in the second dimension, one can observe the pI of a particular protein in a complex mixture. The pI values estimated using Polybuffer exchanger are not in agreement with the results from isoelectro-focusing. It can be explained by a displacement effect and also by nonspecific interaction with PBE 94. Owens *et al.* [16] reported a pI range of 5.5–6.0 for β-glucuronidase from rat liver lysosomal extract by LKB 110 isoelectric focusing column in 6 M urea which was then confirmed by polyacrylamide isoelectric focusing. Also Tomino *et al.* [20] reported the multiplicity of β-glucuronidase from mouse liver lysosomal fraction (about 12 forms) using electrofocusing in a sucrose gradient. Our results obtained by a highly sensitive immunotechnique confirmed the presence of multiple β-glucuronidase forms in liver lysosomal fractions.

Interaction of β-glucuronidase with lectins

The interactions between β-glucuronidase and different lectins were examined using two dimensional and rocket immunoelectrophoresis and the results are summarized in Table 2. Two dimensional immunoelectrophoresis of Con A-Sepharose affinity purified material from a crude lysosomal extract with monospecific anti β-glucuronidase antiserum gave a single precipitation peak (Fig. 5, control). Inclusion of Con A, LCA, WGA, SBA, PAL, DBA or LTL into the first dimension gel led to the appearance of several peaks or the retardation of the peak observed in the control, due to the interaction between lectin and glycan (Fig. 5, Table 2). The results obtained with SBA, DBA and LTL were identical, while those obtained with the remaining lectins were specific for each lectin.

Rocket immunoelectrophoresis of purified β-glucuronidase from lysosomal and microsomal fractions is shown in Figs 6(a), 7(a), respectively. As a result of the reaction with the lectin, a partial decrease of the rocket surface or complete rocket disappearance can be observed. A concentration of 50 µg of lectin per well was chosen as the most effective in reaction with β-glucuronidase. The specificity of most of the lectins used in this study is well known, and therefore the results obtained can be used to predict structures of the glycans present on the enzyme.

Con A. During the purification procedure, all the β-glucuronidase activity was strongly adsorbed to a Con A-Sepharose column (elution with 0.5 M α-methyl-D-glucoside). In two dimensional immunoelectrophoresis, the enzyme was partially retarded in Con A-agarose gel (Fig. 5), whereas in rocket immunoelectrophoresis β-glucuronidase both of lysosomal and microsomal origin reacted strongly with Con A, causing complete disappearance of the rocket (Figs 6(b), 7(b)). The partial reaction in two dimensional immunoelectrophoresis in comparison to total reaction in rocket immunoelectrophoresis may be due to the different concentrations of Con A used in the two techniques or to different pH's (pH 8.8 and pH 6.8, respectively). These results suggest the presence of N-linked glycans of the oligomannosidic, hybrid and/or complex, bi-antennary type [24–28].

LCA, PSA. Interaction of β-glucuronidase with LCA resulted in partial retardation and changing of peak shape. This is in agreement with the quantitative results obtained in rocket immunoelectrophoresis where 67% of lysosomal and 87% of microsomal β-glucuronidase molecules reacted with LCA (Figs 6(c), 7(c)). The presence of bi-antennary fucosylated complex structures is also confirmed by the reaction of 11% and 27% of β-glucuronidase molecules (lysosomal and microsomal forms, respectively) with PSA (Figs 6(d), 7(d)).

WGA. Partial retardation and changing of peak shape obtained with the two dimensional technique indicate the existence of hybrid or complex type glycans with bisecting

Table 2. Summary of results obtained by two dimensional and rocket affino-immunoelectrophoresis of lysosomal (A) and microsomal (B) β-glucuronidases.

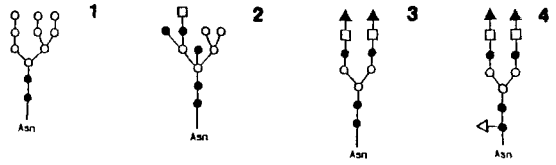
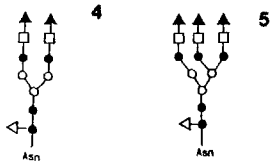
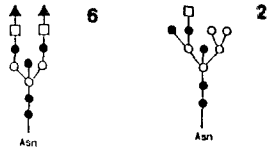
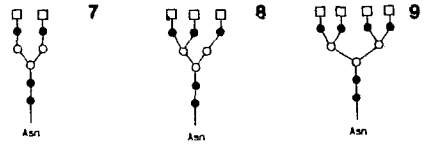
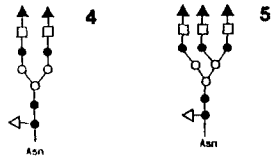
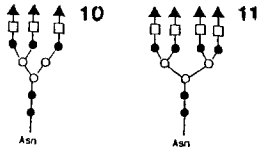
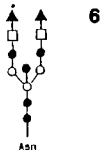
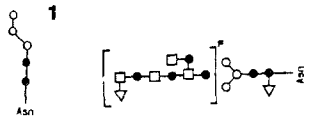
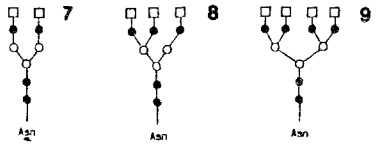
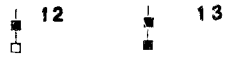
Lectin	*Interaction in immunoelectrophoresis*			*Main specificity*	*References*
	Two dimensional	*Rocket*			
		A	*B*[a]		
Con A	+	100	100		24–28
LCA	+	67	87		25, 28
PSA	N.D.	11	27		25, 28
WGA	+	22	0		24, 25
RCA_I	N.D	16	27		24, 25, 30
LTL	+	11	0		25, 27
PHA-P	−	0	0	Mixture of isolectins E and L	24, 29
PHA-L	N.D.	0	0		24, 29
PHA-E	N.D.	0	0		24, 29
SBA	+	0	0		28
HPA	−	11	10		28
DBA	+	0	0		28

Table 2 (*continued*)

Lectin	*Interaction in immunoelectrophoresis*			*Main specificity*	*References*
	Two dimensional	*Rocket*			
		A	*B*[a]		
PAL	+	0	0		24, 28
MLA	N.D.	11	10	■ 15	30
ECA	N.D.	11	10		24, 25, 30
CAA	N.D.	0	0		28

[a] Percentage of rocket weight in comparison to the weight of control rocket. Compounds are represented by symbolic notation: NeuAc, ▲; Fuc, △; GalNAc, ■; Gal, □; GlcNAc, ●; Man, ○; Asn, asparagine. Glycan types: 1, oligomannosidic; 2, hybrid; 3, complex bi-antennary; 4, complex bi-antennary fucosylated; 5, complex tri-antennary fucosylated; 6, complex bi-antennary type with bisecting *N*-acetylglucosamine; 7, asialo complex bi-antennary; 8, asialo complex tri-antennary; 9, asialo complex tetra-antennary; 10, complex tri-antennary; 11, complex tetra-antennary; 12, GalNAcα(1-3)Gal; 13, GalNAcα(1-3)GalNAc; 14, poly(*N*-acetyllactosamine); 15, *N*-acetylgalactosamine.
N.D., not determined.

N-acetylglucosamine structures (Fig. 5). In rocket electrophoresis, 22% of lysosomal β-glucuronidase reacted with WGA while no reaction with the microsomal enzyme was observed (Figs 6e, 7e).

PHA. β-Glucuronidase did not react in two dimensional immunoelectrophoresis with PHA-P (which is a mixture of two isolectins, PHA-L and PHA-E) (Fig. 5). The same results were obtained by rocket immunoelectrophoresis, where PHA-P, PHA-L and PHA-E were separately used in reaction with both the lysosomal and the microsomal enzyme (Figs 6h–j, 7h–j).

LTL. Strong reaction with LTL resulting in peak retardation in the two dimensional technique, provides evidence that β-glucuronidase possesses glycans with fucose residues α(1-6)-linked to the innermost *N*-acetylglucosamine residue [25, 27]. In rocket immunoelectrophoresis, 11% of lysosomal β-glucuronidase reacted with this lectin (Fig. 6g) while the microsomal enzyme did not react under these conditions (Fig. 7g).

HPA, SBA, DBA, CAA. The results obtained with HPA, SBA, DBA and CAA need very careful interpretation. We observed considerable retardation of the β-glucuronidase peak with SBA and DBA, while with HPA the pattern was not changed under two dimensional immunoelectrophoresis conditions (Fig. 5). In rocket immunoelectrophoresis with SBA, DBA and CAA no reaction was observed for both forms of β-glucuronidase (Figs 6k, m, r and 7k, m, r). In the case of HPA only 11% of lysosomal and 10% of microsomal β-glucuronidase reacted in these conditions (Figs 6l, 7l).

RCA_I, ECA. 16% of lysosomal and 27% of microsomal β-glucuronidase molecules reacted with RCA_I in rocket immunoelectrophoresis conditions (Figs 6f, 7f), whereas ECA interacted with 11% of lysosomal and 10% of microsomal forms.

PAL. In two dimensional immunoelectrophoresis the β-glucuronidase reacted with PAL giving a peak similar to the control but migrating faster (Fig. 5). In the second technique the surface of the rocket was unchanged when compared to the control. At the same time the rocket became higher and narrower (Figs 6n, 7n).

The results presented here provide new information on the reaction of rat liver β-glucuronidase with lectins. The sugar specificity of many lectins is already well established (see Table 2) and therefore the results obtained can be used in order to suggest structures of β-glucuronidase glycans. However, it should be kept in mind that the interpretation of the results of affinity chromatography with immobilized lectin applied for lectin affinity electrophoresis with free lectin cannot be without reservation. Also minor differences observed by weight of rocket area are only of qualitative value.

1. The presence of bi-antennary complex, and/or oligomannosidic type and/or hybrid type is indicated by the reaction with Con A, whereas the absence of the tri-, tetra-antennary complex type glycans is demonstrated by the lack of reaction with PHA-L.

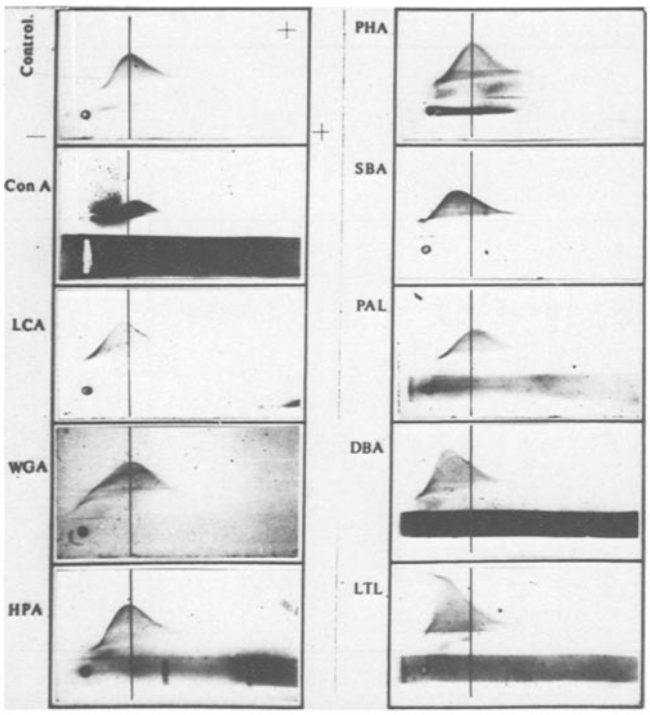


Figure 5. Lectin affinity patterns of β-glucuronidase from rat liver lysosomal fraction. 7.5 µl of sample was separated in 1% agarose containing different lectins (300 µg Con A per cm^2 and 50 µg other lectins per cm^2) for 2 h at 10 V cm^{-1}. Immunoelectrophoresis was performed in 1% agarose containing 0.9 µl cm^{-2} rabbit anti-glucuronidase serum for 18 h at 2 V cm^{-1}. The vertical line indicates the position of the peak in the control. For full experimental details see the Materials and methods section.

2. The presence of a hybrid type and the absence of bi-antennary complex type glycans containing a bisecting *N*-acetylglucosamine residue in the lysosomal form of β-glucuronidase was confirmed by the partially positive reaction with WGA and negative reaction with PHA-E, respectively. Microsomal forms seemed not to possess hybrid type glycan because no reaction with WGA was observed.

3. Additional information is provided by applying LCA and PSA. These lectins have the same specificity and, apart from the mannose core, require the presence of a fucose residue α(1-6)-linked to the innermost *N*-acetylglucosamine attached to asparagine. Based on our results it appears that only part of the bi-antennary complex type glycans is fucosylated. Microsomal β-glucuronidase contains more fucosylated variants than the lysosomal enzyme.

4. Stronger reaction of microsomal β-glucuronidase with RCA_I when compared to the lysosomal form might suggest that the former possesses more exposed, terminal non-substituted Galβ(1-4) residues.

5. The interaction between lysosomal β-glucuronidase and LTL might also be due to an "avidity effect" caused by the presence of the additional α(1-3)-fucose residues substituting the antennary *N*-acetylglucosamine residues. For better characterization of the fucose substitution, the lectin from the mushroom *Aleuria aurantia* and from *Ulex europeus* could be useful.

6. The results obtained do not answer the question concerning the number of glycans on the molecule of β-glucuronidase. Therefore sequential fractionation of isolated glycans by the use of immobilized lectins seems to be necessary. Nevertheless, our results demonstrate that the lectin affinity immunoelectrophoretical methods are useful for elucidation of the microheterogeneity of glycoproteins, although for structure determination use of other techniques is also required.

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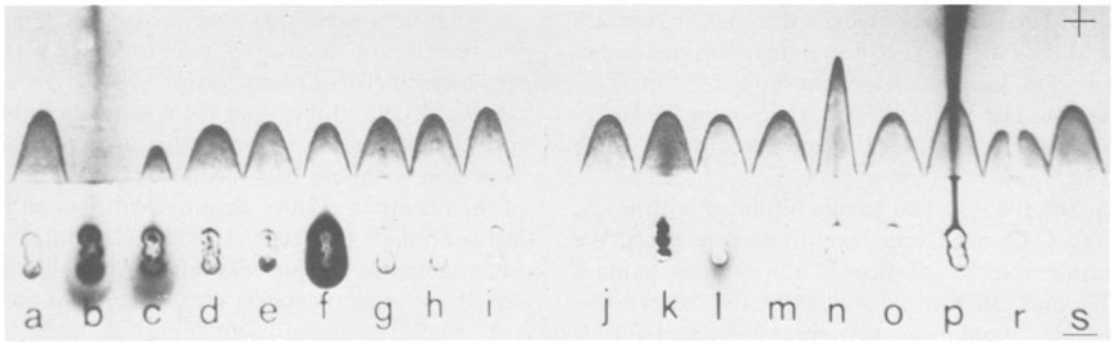

Figure 6. Rocket immunoelectrophoresis of crude lysosomal extract (20 µl). Each well contained 50 µg of appropriate lectin: (a) and (s), controls; (b), Con A; (c), LCA; (d), PSA; (e), WGA; (f), RCA_I; (g), LTL; (h), PHA-P; (i), PHA-L; (j), PHA-E; (k), SBA; (l), HPA; (m), DBA; (n), PAL; (o), MLA; (p), ECA; (r), CAA. Electrophoresis was performed in 1% agarose containing 0.9 µl cm^{-2} rabbit antiglucuronidase serum for 19 h at 2 V cm^{-1}.

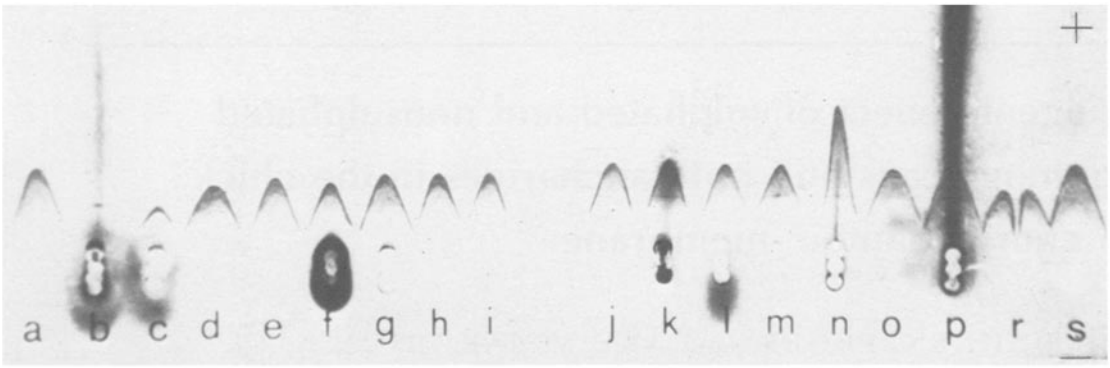

Figure 7. Rocket immunoelectrophoresis of crude microsomal extract (35 µl). Order of lectins and electrophoretical conditions as in Fig. 6.

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