BBA 66162

THE PURIFICATION OF LYSOSOMAL RAT-LIVER β-GLUCURONIDASE*

E. DELVIN** AND R. GIANETTO
Département de Biochimie, Université de Montréal, Montréal (Canada)

(Received May 4th, 1970)

SUMMARY

β-Glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) from rat-liver lysosomes was purified by $(NH_4)_2SO_4$ precipitation, gel filtration on Sephadex G-200, treatment with trypsin, and fractionated into two active components by ion-exchange chromatography on DEAE-Sephadex A-25. The two components had the same molecular weight, 250 000 ± 15 000 (S.E.) and the same Stokes radius 73.7 ± 3.1 Å (S.E.). Their electrophoretic mobilities on polyacrylamide gel were however slightly different. K_m determinations for each component yielded similar results at two pH values.

INTRODUCTION

The presence of multiple forms of β-glucuronidase in mammalian tissues has been subject to controversy since 1948 when MILLS[1] isolated from bovine spleen two β-glucuronidases having different pH optima and kinetic characteristics. These results were questioned by BERNFELD AND FISHMAN[2-4] who suggested that the different forms of β-glucuronidase might be the result of complexes of the enzyme with various tissue constituents.

DEDUVE *et al.*[5] have shown by tissue fractionation studies that the β-glucuronidase of rat liver has a dual localization being contained in the lysosomal fraction and in the microsomal fraction, although the former fraction contains a higher percentage of the enzyme. These results were confirmed by FISHMAN *et al.*[6] using cytochemical techniques. However no attempts were made to purify the enzyme from these subcellular fractions.

This report presents a method for the purification of rat-liver lysosomal β-glucuronidase and its separation into two active components.

MATERIAL AND METHODS

Phenolphthalein β-D-glucuronide, glucose 6-phosphate, Fast Garnet GBC and

* From the Ph. D. Thesis of E. Delvin, Université de Montréal, Montréal, Canada.
** Present address: The deBelle Laboratory for Biochemical Genetics, McGill University, Montreal Children's Hospital.

8-hydroxyquinoline β-D-glucuronide were purchased from Sigma Chemical Co. Trypsin and soya bean trypsin inhibitor were obtained from Worthington Biochemical Corporation. Sephadex G-25, Sephadex G-200, DEAE-Sephadex A-25, and Blue Dextran 2000 were purchased from Pharmacia. The proteins used as molecular weight markers were from Mann Research Laboratories.

Enzyme assays

β-Glucuronidase activity was determined as described by GIANETTO AND DEDUVE[7]. One unit of β-glucuronidase activity is the amount of enzyme that liberates one μmole of phenolphthalein per min at 37°.

Glucose-6-phosphatase activity was measured by the method of DEDUVE *et al.*[5]. Suitable blanks were made in all cases.

Protein determinations

Protein concentrations were estimated by the ultraviolet absorption method of WARBURG AND CHRISTIAN[8].

Purification procedure

Livers weighing 453 g were obtained from male Wistar rats (125–150 g) that had been fasted for 16 h before being killed by a blow on the head and bled. They were then homogenized in batches of 15–18 g in 0.25 M sucrose with a Potter–Elvehjem homogenizer fitted with a Teflon pestle and fractionated according to the method of DEDUVE *et al.*[5], except that the mitochondrial and lysosomal fractions were centrifuged together at 29 700 × *g* during 10 min in the rotor A-170 of the International ultracentrifuge, Model B-60, and washed once with 0.25 M sucrose. The pellet containing mitochondria and lysosomes was resuspended in distilled water (2.5 ml for each g of liver, *i.e.* 1133 ml for 453 g), treated during 1.5 min in a precooled Waring Blendor, and centrifuged at 105 000 × *g* during 40 min. This centrifugation eliminated the lysosomal membranes and the mitochondrial debris as well as most of the contaminating microsomes.

The clear supernatant was diluted with distilled water so as to have the equivalent of 1 g of liver per 10 ml of solution. The pH was adjusted to 5.5 with dilute HCl and brought to 40% saturation with $(NH_4)_2SO_4$ (solid) at 4°. After 8 h the suspension was centrifuged and the clear supernatant brought to 70% saturation with $(NH_4)_2SO_4$. The precipitated proteins were centrifuged after 16 h and dissolved in a minimum volume of 2 mM Tris buffer (pH 7.3), containing 5 mM NaCl. Insoluble material was eliminated by centrifugation. The resulting solution was freed of $(NH_4)_2SO_4$ by gel filtration on Sephadex G-25 previously equilibrated with the Tris–NaCl buffer mentioned above.

The fractions containing the β-glucuronidase activity were pooled and incubated at 37° with 0.02% trypsin in the presence of 0.01 M phosphate buffer (pH 7.6). The pH was kept constant during the incubation by adding dilute NaOH. Two subsequent additions of trypsin equal to the initial amount were made at 30-min intervals. After 1.5 h of proteolysis an equivalent amount of soya bean trypsin inhibitor was added to the incubation mixture which was then brought to 70% saturation with $(NH_4)_2SO_4$ and left standing at 4° overnight before being centrifuged. The precipitated proteins were dissolved in a minimum volume of Tris–NaCl buffer (pH 7.3), mentioned

above, and chromatographed on a Sephadex G-200 column previously equilibrated with the same buffer. The fractions containing the β-glucuronidase activity were pooled and concentrated by ultrafiltration on an Amicon XM-50 membrane to a volume of 10 ml.

The concentrated solution was then chromatographed on a DEAE-Sephadex A-25 column previously equilibrated with 0.01 M glycylglycine buffer (pH 7.0) containing 0.1 M NaCl. The β-glucuronidase activity was eluted into two components with the same buffer, except that a NaCl gradient was started after elution of the first component. The fractions from each peak containing the enzymatic activity were pooled, concentrated by ultrafiltration on an Amicon XM-50 membrane, and rechromatographed on DEAE-Sephadex A-25 under the same conditions as above.

Disc electrophoresis

Disc electrophoresis was performed on polyacrylamide gel according to the procedure of DAVIS[9], except that the 7.5% separating gel was replaced by a 5% gel. The β-glucuronidase activity after electrophoresis was detected by the following procedure. The gel was incubated for 30 min at 37° in 0.1 M acetate buffer (pH 5.0), containing 0.6 M NaCl, then placed in a tube containing the same buffer with 3 mM 8-hydroxyquinoline β-D-glucuronide as the substrate and Fast Garnet GBC, 1 mg/ml, as the diazo coupling agent, and incubated at 37° during 1 h. The enzyme was revealed by a brown band. Proteins were detected by the Amido-black method followed by destaining in 7% acetic acid.

Molecular weight and Stokes radius

Sephadex G-200 was used to evaluate the molecular weight and the Stokes radius of the purified lysosomal β-glucuronidase. The column was calibrated with proteins of known molecular weight using the Tris–NaCl buffer mentioned earlier. Protein concentration in the eluate was measured by absorbance at 230 nm except catalase which was measured by the method of CHANCE AND MAEHLY[10]. The elution volumes (V_e) were measured at the maximum of the absorbance peaks. The void volume (V_0) and the inner volume (V_i) of the column were measured with Blue Dextran 2000 and $(NH_4)_2SO_4$, respectively.

RESULTS

Since the microsomal fraction of rat liver contains some β-glucuronidase activity[5,6], contamination of the lysosomal fraction by microsomes was evaluated by measuring its content in glucose-6-phosphatase which is essentially a microsomal enzyme[11]. The lysosomal fraction contained less than 3% of the glucose-6-phosphatase activity of the whole homogenate. Since microsomes contain from 20 to 30% of the β-glucuronidase activity[12], the lysosomal β-glucuronidase could not have been contaminated by more than 1% with microsomal β-glucuronidase.

The centrifugation pellet containing the lysosomes accounted for 53% of the β-glucuronidase activity of the nuclei-free homogenate. No activity was lost after dilution of this pellet in water, treatment in the Waring Blendor, and centrifugation at 105 000 × *g* during 40 min.

TABLE I

PERCENTAGE OF INITIAL β-GLUCURONIDASE ACTIVITY AFTER TREATMENT WITH TRYPSIN FOR INCREASING PERIODS OF TIME

	Time (min)			
	0	*10*	*30*	*120*
β-Glucuronidase + trypsin	100	102	107	106
β-Glucuronidase − trypsin	100	100	99	102

Precipitation by $(NH_4)_2SO_4$ and chromatography on Sephadex G-25 were performed without appreciable loss of activity.

Proteolysis by trypsin does not inactivate the β-glucuronidase obtained from the preceeding steps as shown in Table I. When the β-glucuronidase is not incubated

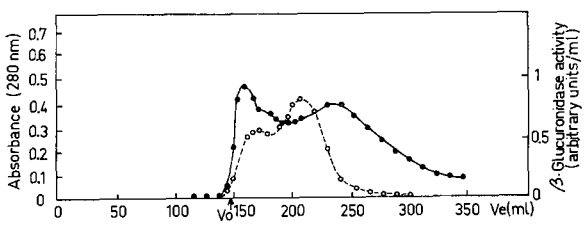

Fig. 1. Gel filtration on a Sephadex G-200 column (2.5 cm × 90 cm) of the β-glucuronidase activity precipitated between 40 and 70% $(NH_4)_2SO_4$ saturation. Equilibration of the column and elution were performed with 2 mM Tris buffer (pH 7.3) containing 5 mM NaCl. Void volume, V_0; elution volume, V_e; proteins, ●—●; β-glucuronidase activity, ○ — — ○.

with trypsin before being chromatographed on Sephadex G-200, it is eluted with relatively large amounts of proteins as shown by Fig. 1. After tryptic digestion most of the proteins have been transformed into substances of lower molecular weights and a greater purification of the enzyme is obtained as shown by Fig. 2. Recovery of the β-glucuronidase activity for these two last steps was 90%.

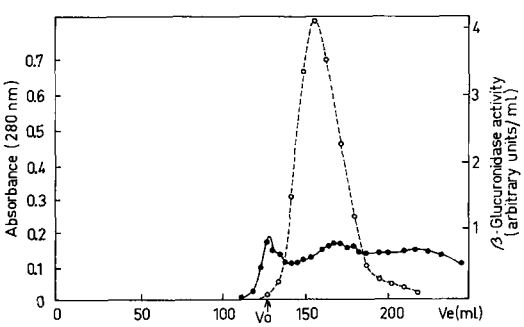

Fig. 2. Gel filtration on a Sephadex G-200 column (2.5 cm × 78 cm) of the β-glucuronidase activity precipitated between 40 and 70% $(NH_4)_2SO_4$ saturation and treated with trypsin. Equilibration of the column and elution were performed with 2 mM Tris buffer (pH 7.3) containing 5 mM NaCl. Void volume, V_0; elution volume, V_e; proteins, ●—●; β-glucuronidase activity, ○ — — ○.

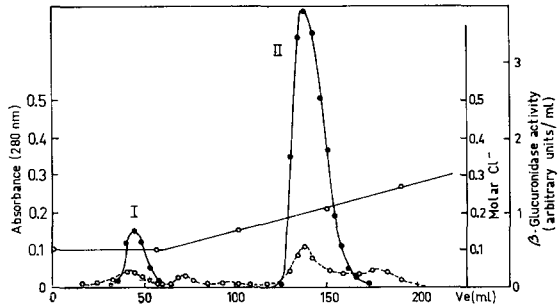

Fig. 3. Chromatography on a DEAE-Sephadex A-25 column (1 cm × 58 cm) equilibrated with 0.01 M glycylglycine buffer (pH 7.0), containing 0.1 M NaCl of β-glucuronidase treated with trypsin. Proteins, ○ – – ○; β-glucuronidase, ●—●; NaCl gradient, ○——○.

Chromatography on DEAE-Sephadex A-25 was performed with a 90% recovery of the enzyme. As shown by Fig. 3, the β-glucuronidase activity was eluted into two components, the first one accounting for 12% and the second for 78% of the activity deposited on the column. The elution of the β-glucuronidase activity into two components is not an artifact resulting from the digestion by trypsin since chromatography of a β-glucuronidase preparation not treated with trypsin also gives two peaks of activity as shown by Fig. 4.

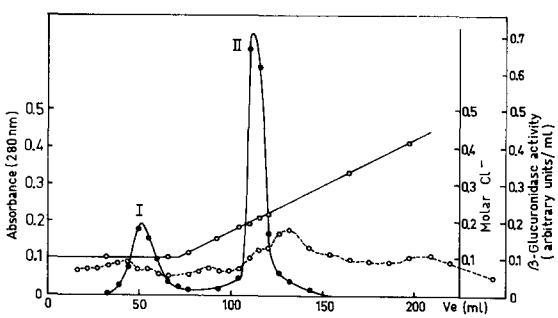

Fig. 4. Chromatography on a DEAE-Sephadex A-25 column (1 cm × 58 cm) equilibrated with 0.01 M glycylglycine buffer (pH 7.0) containing 0.1 M NaCl of β-glucuronidase not treated with trypsin. Proteins, ○ – – ○; β-glucuronidase, ●—●; NaCl gradient, ○—○.

When the two peaks of activity were rechromatographed separately, each of them was eluted at the same volume and NaCl concentration as it was initially eluted. Only a very small percentage, less than 1%, of the activity of Peak I was eluted at the volume and NaCl concentration of Peak II and *vice versa*. Table II summarizes the purification procedure steps.

We have shown that the two β-glucuronidase components have similar pH–activity curves, both showing optimum activity at two pH values, namely 4.7 and 5.3 (ref. 13). The Michaelis–Menten constants were thus determined at these two pH values in the presence of phenolphthalein β-D-glucuronide, concentration range 0.125–15 mM. For the first component at pH 4.7, K_m = 44 μM, and at pH 5.3, K_m = 120 μM. For the second component the respective values were 48 and 110 μM.

TABLE II

PURIFICATION STEPS OF RAT-LIVER LYSOSOMAL β-GLUCURONIDASE

	Total units		*Proteins (mg)*	*Specific activity (units/mg)*	*Recovery (%)*	
Nuclei-free homogenate	506		79547	0.006	100	
Lysosomal-enriched fraction	268		21925	0.012	53	
Waring Blendor treatment and centrifugation at 105 000 × *g* during 40 min	268		4258	0.063	53	
40–70% $(NH_4)_2SO_4$ saturation and gel filtration on Sephadex G-25	268		1721	0.156	53	
Trypsin treatment and gel filtration on Sephadex G-200	238		154	1.54	47	
Chromatography on DEAE-Sephadex A-25	Peak I 28 Peak II 186	214	5.9 28.5	4.75 6.53	5.5 36.8	42.3

Disc electrophoresis followed by detection of enzymatic activity, as described under MATERIAL AND METHODS, was performed on each of the two peaks of activity. Peak II migrated slightly faster than Peak I. When equal amounts of Peak I and Peak II were treated together, only one large band was obtained which extended from the upper limit of the band given by Peak I to the lower limit of the band given by Peak II. When the polyacrylamide gels were stained for proteins, both peaks were shown to contain four contaminating proteins besides the major protein band which corresponded to the enzymatic activity.

Fig. 5 shows the relationship between the log molecular weight and the elution volume over void volume (V_e/V_0) for several globular proteins chromatographed on Sephadex G-200. Each point is the mean V_e/V_0 of three determinations. V_e/V_0 was the same for both β-glucuronidases. If it is assumed that they are globular proteins, the estimated molecular weight is 250 000 ± 15 000 (S.E.).

The Stokes radius of the two β-glucuronidases was calculated according to the method of ACKERS[14] using the Sephadex G-200 data. Stokes radii of the standard

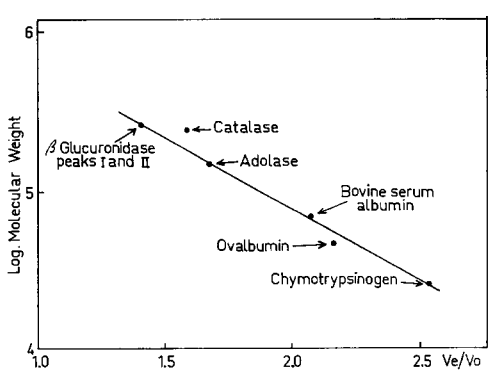

Fig. 5. Estimation of the molecular weight of rat-liver lysosomal β-glucuronidases on a Sephadex G-200 column (2.5 cm × 72 cm). V_e is the elution volume, V_0 is the void volume. The equilibration and elution buffer was 2 mM Tris (pH 7.3) containing 5 mM NaCl. The method of least squares was used for drawing the line.

reference proteins were found in the literature for bovine serum albumin[15] and ovalbumin[16], and calculated from the diffusion coefficients for catalase[17] and aldolase[16]. The Stokes radius of the two β-glucuronidases was found to be 73.7 ± 3.1 Å (S.E.), assuming that they have a spherical shape.

DISCUSSION

Our results show that the two β-glucuronidases purified from rat-liver lysosomes and separated by chromatography on DEAE-Sephadex A-25, differ only by their electric charge because other properties such as pH optima, molecular weights, and K_m values were the same. Similar properties have been reported for other rat-liver lysosomal enzymes. BECK *et al.*[18] have shown that the two acid phosphatases from rat-liver lysosomes, separated by chromatography on CM-cellulose, have the same pH–activity curves in the presence of β-glycerophosphate. BRIGHTWELL AND TAPPEL[19] have reported that acid pyrophosphatase was resolved by chromatography on DEAE-cellulose into two components that have the same pH–activity curves and same K_m values in the presence of ATP.

We have shown that trypsin is without effect on the lysosomal β-glucuronidase activity of rat-liver or on its elution profile on DEAE-Sephadex A-25. According to several authors[20–22], the resistance to trypsin is a property shared by several glycoproteins. Unpublished results from our laboratory indicate that rat-liver β-glucuronidases are glycoproteins. Bovine-liver β-glucuronidases have also been shown to be glycoproteins by PLAPP AND COLE[23]. One may then wonder if the difference in electric charge of the two β-glucuronidases is due to a different content of glucidic residues such as glucosamine and sialic acid or to a different amino acid sequence or to both.

ACKNOWLEDGMENTS

This research was supported by a grant from the Medical Research Council of Canada. We would like to thank the Department of Health of the Province of Quebec for its financial support to E. D..

REFERENCES

1 G. J. MILLS, *Biochem. J.*, 43 (1948) 125.
2 P. BERNFELD AND W. H. FISHMAN, *Arch. Biochem.*, 27 (1950) 475.
3 P. BERNFELD AND W. H. FISHMAN, *J. Biol. Chem.*, 202 (1953) 757.
4 P. BERNFELD AND W. H. FISHMAN, *J. Biol. Chem.*, 202 (1953) 763.
5 C. DEDUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
6 W. H. FISHMAN, S. S. GOLDMAN AND R. DELELLIS, *Nature*, 213 (1967) 457.
7 R. GIANETTO AND C. DEDUVE, *Biochem. J.*, 59 (1955) 433.
8 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1942) 384.
9 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
10 B. CHANCE AND A. C. MAEHLY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 764.
11 G. HERS, J. BERTHET, L. BERTHET AND C. DEDUVE, *Bull. Soc. Chim. Biol.*, 33 (1951) 21.
12 R. GIANETTO, *Can. J. Biochem.*, 42 (1964) 499.
13 E. DELVIN AND R. GIANETTO, *Can. J. Biochem.*, 46 (1968) 389.
14 G. K. ACKERS, *J. Biol. Chem.*, 242 (1968) 3237.
15 G. K. ACKERS AND R. L. STEERE, *Biochim. Biophys. Acta*, 59 (1962) 137.

16 J. T. EDSALL, in H. NEURATH AND K. BAILEY, *The Proteins*, Vol. 1, Part B, Academic Press, New York, 1953, p. 636.
17 C. TANFORD, *Physical Chemistry of Macromolecules*, John Wiley and Sons, New York, 1967, p. 358.
18 C. BECK, S. MAHADEVAN, R. BRIGHTWELL, C. J. DILLARD AND A. L. TAPPEL, *Arch. Biochem. Biophys.*, 128 (1968) 369.
19 R. BRIGHTWELL AND A. L. TAPPEL, *Arch. Biochem. Biophys.*, 124 (1968) 333.
20 I. YAMASHINA, *Acta Chem. Scand.*, 10 (1956) 1666.
21 E. A. POPENOE AND R. M. DREW, *J. Biol. Chem.*, 228 (1957) 673.
22 A. GOTTSCHALK AND S. FAZEKAS DE ST-GROTH, *Biochim. Biophys. Acta*, 43 (1960) 513.
23 B. V. PLAPP AND R. D. COLE, *Biochemistry*, 6 (1967) 3676.