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Preparation of Crystalline Bovine Liver β-Glucuronidase

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Summary. A method is described for the preparation of pure β-glucuronidase from bovine liver. The procedure includes ammonium sulfate, acetone and ethanol fractionation and a simple two-step ion exchange chromatography. The yield is acceptable and the method requires only standard laboratory equipment. The pure enzyme is easily crystallized from ammonium sulfate. Some practical applications of the pure β-glucuronidase are discussed.

Key word: β-Glucuronidase, Purification Method

Zusammenfassung. Eine Methode für die Reindarstellung von β-Glucuronidase aus Rinderleber wird beschrieben. Es wird eine Ammonsulfat-, Aceton- und Äthanolfraktionierung zusammen mit einer einfachen zweistufigen Ionenaustauschchromatografie verwendet. Die Ausbeute ist annehmbar und die Methode erfordert nur gewöhnliche Laboratoriumsausstattung. Das reine Enzym ist leicht aus Ammonsulfat zu kristallisieren. Es wird über einige praktische Verwendungen der reinen β-Glucuronidase diskutiert.

Schlüsselwörter: β-Glucuronidase, Reinigungsmethode – Arzneimittelmetaboliten

Methods of purifying bovine liver β-glucuronidase (EC 3.2.1.31.) have previously been reported by several investigators (1–4). Plapp and Cole [5] showed that bovine liver β-glucuronidase is a glycoprotein and is microheterogeneous in the sense that multiple forms exist, having similar protein structures but differing slightly in carbohydrate content. Previously, Bonnichsen [2] crystallized purified beef liver β-glucuronidase by dialyzing against distilled water but the material was probably not completely pure. Himeno et al. [4] developed a procedure to obtain highly purified β-glucuronidase which was homogeneous by all criteria tested but they did not crystallize the enzyme and the overall yield was low. Himeno et al. [6] obtained crystalline β-glucuronidase from the female rat preputial gland. In the present paper we describe a simple purification method for bovine liver β-glucuronidase which utilizes acetone, ammonium sulfate and ethanol fractionation and DEAE-Sephadex A-25 chromatography. The enzyme prepared by this method was homogeneous by gel electrophoresis and crystallization.

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Materials and Methods

DEAE-Sephadex A-25 and Agarose A were purchased from Pharmacia, Uppsala, Sweden, DEAE-23 SS cellulose (Servacel ®) from Serva Feinbiochemica, Heidelberg, Germany. Absolute ethanol (99,5 %) was used throughout. Ultra pure ammonium sulfate was used after step 5 in the purification procedure. All other chemicals were of reagent grade, obtained from various commercial sources.

β-Glucuronidase was routinely assayed by the method of Plapp and Cole [3] with phenolphtalein-β-D-glucuronide as substrate. One unit of enzyme activity is defined as the amount of enzyme which liberates one μg of phenolphtalein in one hour under optimal conditions. Specific activity was expressed as units per mg of protein. When p-nitrophenyl-β-D-glucuronide was used as substrate the specific activity was expressed in I. U.

Protein was determined by the method of Lowry et al. [7] with crystalline bovine serum albumin as standard. All centrifugations were performed in MSE (Mistral 6 l) centrifuge giving an RCF of about 700x $g_{av.}$ All chromatographies were performed at room temperature.

Agarose gel electrophoresis was performed on a cooled glass plate at 10°C at pH 7.0 in 0.05 M phosphate buffer or at pH 8.5 in 0.05 M Tris-HCl buffer. The concentration of agarose in the gel was 0.9 %.

Experimental

Method of Purification

Step 1. Extraction. Ground bovine liver (4 kg) was extracted with twice the amount of sodium acetate buffer, pH 5.0 at 4°C overnight and centrifuged next day. [1] All buffers used during the purification contained 1 mM EDTA.

Step 2. Aceton fractionation at 0°C. To one volume of enzyme solution 0.62 volumes of acetone was added. The mixture was centrifuged and the supernatant was discarded. The precipitate was dissolved in about 3 l of 0.1 M sodium phosphate buffer, pH 7.0 and stirred for 15 min. After dialysis against 0.002 M sodium phosphate buffer, pH 7.0 insoluble material was removed by centrifugation.

Step 3. Ammonium sulfate fractionation I. To the enzyme solution solid ammonium sulfate was added at room temperature and the precipitate formed between 37 and 55 % saturation was collected by centrifugation and dissolved in distilled water. After dialysis against 0.05 M sodium acetate buffer, pH 4.4 insoluble material was removed by centrifugation. The volume was now about 500 ml.

Step 4. Ethanol fractionation I. To one volume of the enzyme solution 0.36 volumes of ethanol was added at room temperature. The mixture was centrifuged and the precipitate was discarded. To the supernatant 0.62 volumes of ethanol was added. The collected precipitate was dissolved in 100 ml of 0.1 M sodium acetate buffer, pH 4.4 and dialyzed against 0.001 M sodium phosphate buffer, pH 7.0. Insoluble material was removed by centrifugation.

Step 5. Ammonium sulfate fractionation II. To the enzyme solution solid ammonium sulfate was added at room temperature. The precipitate formed between 40 and 55 % was collected by centrifugation and dissolved in distilled water to a volume of about 30 ml.

Step 6. DEAE Sephadex A-25 chomatography. The enzyme solution containing 300–600 mg protein was dialyzed against 0.001 M sodium phosphate buffer, pH 7.0 and concentrated in a Minicon B-15 clinical sample concentrator at room temperature to 5 ml. The concentrated solution was applied to a 2.5 x 25 cm column of DEAE-Sephadex A-25 previously treated with 0.1 M sodium phosphate buffer, pH 7.0 and finally washed with distilled water. The column was eluted at a rate of 20 ml per hr first with distilled water until no activity was observed in the eluate and then with 120 ml 0.1 M sodium phosphate buffer, pH 7.0. As shown in Figure 1 two peaks of enzyme activity were obtained. The first peak (fraction I) obtained by eluting the column with distilled water contained 30 % of enzyme activity. The second peak (fraction II) obtained with

[1] Occasionally a liver gives a more or less cloudy supernatant. To avoid difficulties in the later stages it is advisable to discard such a preparation and start anew

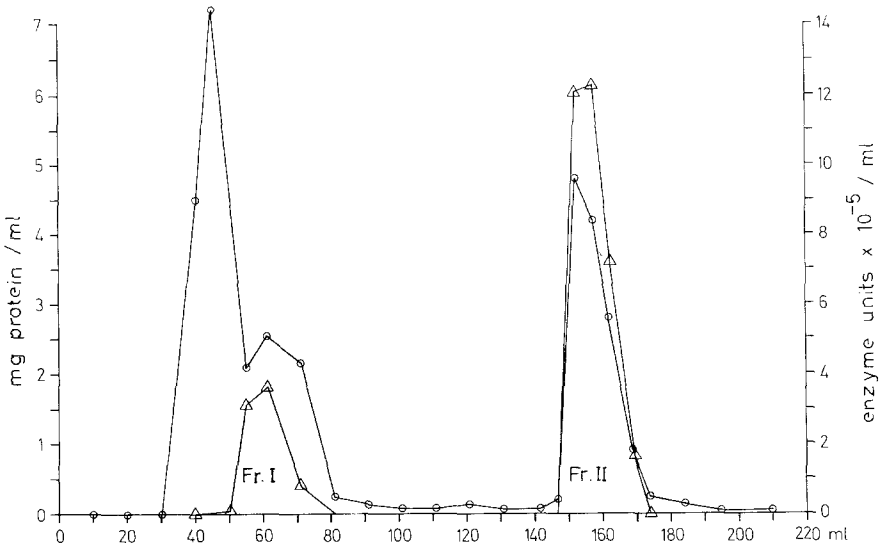

Fig. 1. DEAE-Sephadex A-25 chromatography of β-glucuronidase (step 6): (–△–) enzyme activity; (–○–) protein concentration

0.1 M sodium phosphate buffer, pH 7.0 contained 70 % of enzyme activity and was used for further purification. The specific activity based on protein content, was identical in the two fractions.

Step 7. Ethanol fractionation II at 0°C. Fraction II from step 6 was dialyzed against 0.1 M sodium acetate buffer, pH 5.0 and concentrated in a Minicon B-15. To one volume of enzyme solution (7 ml) containing 12 mg protein per ml 0.19 volumes of ethanol was added. The precipitate was removed by centrifugation and discarded. To the supernatant 0.1 volume of ehtanol was added. The precipitate was collected by centrifugation and dissolved in 0.1 M sodium acetate buffer, pH 5.0. After this step the overall purification was 3900 fold based on the original extract and the specific activity 41.6 I. U. with p-nitrophenyl-β-D-glucuronide as substrate. The total yield was 26 %.

Step 8. Crystallization. The solution from step 7 was dialyzed against 0.2 M sodium phosphate buffer, pH 7.0. To one volume of enzyme solution (30 mg protein per ml) at room temperature was added 0.43 volumes of saturated ammonium sulfate in 0.2 M sodium phosphate buffer, pH 7.0. After standing at 4°C for two weeks the crystallization was complete. The crystals were collected by centrifugation and dissolved in 0.1 M acetate buffer, pH 5.0. The yield was 96 %. A summary of the purification scheme is shown in Table 1.

Results and Discussion

Gel electrophoresis of the purified β-glucuronidase from step 7 on agarose A showed only a single band with two different buffer systems, 0.05 M sodium phosphate buffer, pH 7.0 and 0.05 M Tris-HCl buffer, pH 8.6.

The needle like crystals obtained by the procedure described in step 8 were homogenous in the microscope. A photomicrograph of the crystals is shown in Figure 2. As seen in Table I the crystallization step does not improve the purity and is thus unnecessary for practical purpose.

Rechromatography of fraction II from step 6 gave an unexpected result. An aliquote of this fraction was concentrated with ammonium sulfate and the precipitated

Table 1. Summary of the enzyme purification procedure

Step	Procedure	Enzyme activity (units x 10^3)	Protein (g)	Specific activity (units/mg)	Yield (%)	Purification ratio
1.	Centrifuged supernatant	31,950	328.6	84	100	1
2.	Aceton fractionation	30,800	105.2	293	96.4	3.5
3.	Ammonium sulfate fractionation I	30,800	34.3	898	96.4	10.8
4.	Ethanol fractionation I	19,100	1.95	9,797	60.0	117
5.	Ammonium sulfate fractionation II	19,100	1.10	17,367	60.0	208
6.	DEAE Sephadex A-25 chromatography	11,950	0.085	141,000	37.0	1,687
7.	Ethanol fractionation II	8,280	0.025	330,000 (41.6)[a]	25.9	3,952
8.	Crystallization	7,920	0.024	330,000 (41.6)[a]	24.8	3,952

[a] The numbers in parenthesis represent specific activities as the number of μmoles p-nitrophenol liberated in 1 min under conditions described by Himeno et al. [6] using p-nitrophenyl-β-D-glucuronide as a substrate

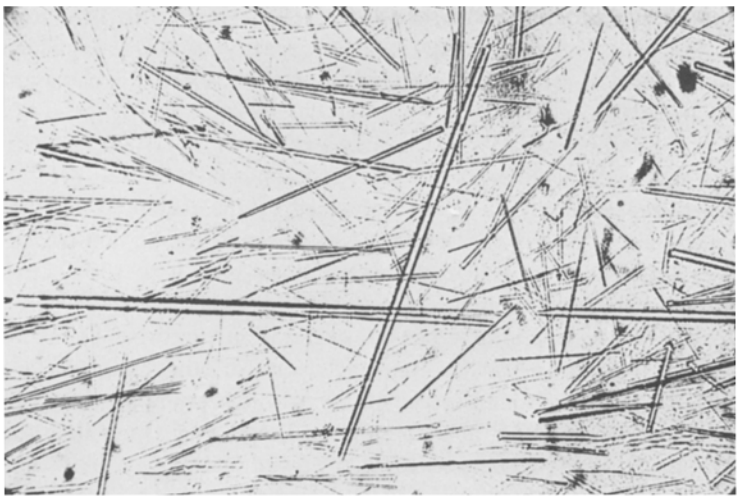

Fig. 2. Crystals of β-glucuronidase crystallized with ammonium sulfate in 0.2 M sodium phosphate buffer, pH 7.0. Magnification 100x

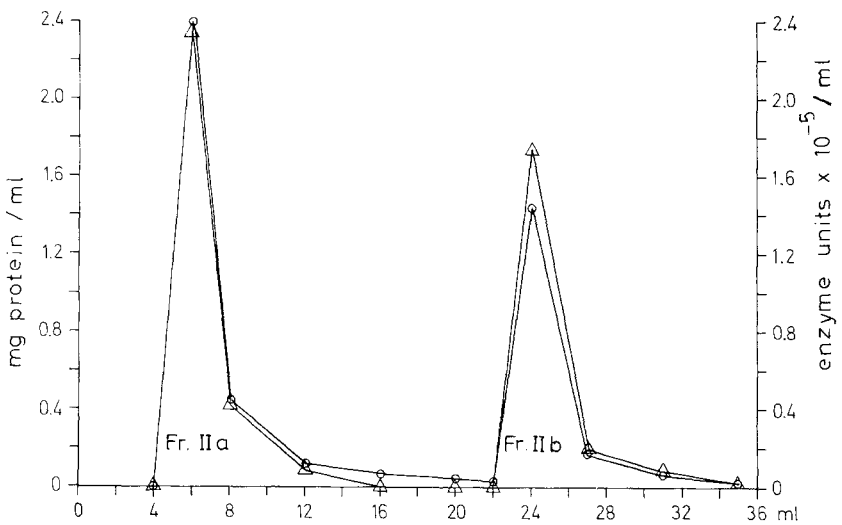

Fig. 3. Rechromatography of fraction II from step 6 on DEAE-Sephadex A-25: (–△–) enzyme activity: (–○–) protein concentration

enzyme dissolved in a minimum volume of 0.1 M sodium acetate buffer, pH 5.0. After (unintentional) storage at 4°C for about two weeks the solution was dialyzed and rechromatographed on DEAE-Sephadex A-25 (Fig. 3) as described in step 6. Again, two peaks of enzyme activity was obtained containing respectively 60 and 40 % of the activity. The specific activity, based on protein content, was identical in the two fractions and the same as the activity of the two fractions in step 6. If, however, the same enzyme solution was rechromatographed on DEAE-23 SS (Servacel ®) cellulose under the same conditions, no peak was obtained by elution with distilled water, a

single peak being obtained with 0.1 M sodium phosphate buffer, pH 7.0. The yield, however, was only 55 %. Considering the much larger capacity of DEAE-Sephadex compared to Servacel a loss of carbohydrate seems the most reasonable explanation of this somewhat contradictory behaviour.

On one occasion the remainder after the acetate extraction at pH 5.0 and centrifugation in step 1 was reextracted with 0.1 M sodium phosphate buffer, pH 7.4. This extract contained one third of the activity obtained by the acetate extraction. After purification steps 2–5 this enzyme solution was chromatographed on DEAE-Sephadex A-25 gel as described in step 6. In this case a single peak with 100 % yield was obtained by elution with distilled water.

Metabolites from a large number of drugs are excreted in the urine bound to different substances such as glucuronic acid, sulfate and glycine or other amino acids.

In order to isolate metabolites bound to glucuronic acid it is essential to have an enzyme preparation free from enzymes able to split off metabolites other than the ones bound to glucuronic acid. The glucuronidase in all probability binds the metabolites which it splits off from glucuronic acid. If these substances are fluorescent, it is also probable that the fluorescence intensity and/or wave length maximum changes on coupling to the enzyme, which would provide a means of identification of these substances. As spectrofluorimetry is an extremely sensitive method, it should be possible to determine very small amounts of material.

The method of preparation of glucuronidase described in this paper is very simple and quite reproducible and the necessary equipment is found in almost every laboratory. The yield is fairly good and the cost and labour involved is small compared to the cost for impure (commercial) preparations.

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