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Purification and properties of beef heart β-glucuronidase

β-Glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3 2 1.31) is an acid hydrolase localized in the lysosomes and the endoplasmic reticulum[1] Research carried out in this laboratory has shown that β-glucuronidase, together with other acid hydrolases, is present in beef heart and biochemical evidence has indicated that it is associated with lysosomes[2] The enzyme presumably functions in the catabolic breakdown of mucopolysaccharides, although it may also be involved in synthetic processes[3] Since the early attempts to purify β-glucuronidase from ox spleen[3] many procedures have been developed to isolate the enzyme from a variety of tissues[3,4–6] In this communication a procedure is reported for solubilization and purification of β-glucuronidase from beef myocardium Some properties of the purified enzyme are also reported

Enzyme activity was determined by incubating appropriately diluted solutions of the enzyme for 60 min at 37° in a reaction mixture (1 ml) containing 0.5 mM phenolphthalein β-glucuronide (Sigma, U S A), 0 1 M acetate buffer (pH 5 2) and 0.1% bovine serum albumin (Sigma) (Unless indicated, all the assays with inhibitors were run without albumin) The reaction was stopped by the addition of 3 ml of 0 133 M glycine–NaOH buffer (pH 10 45) and phenolphthalein measured at 550 mμ. Protein was determined by the method of WADDELL[7] Albumin was used as standard

All purification steps were done at 2–5° The reagent solutions were prepared with distilled water deionized through Dowex 50 (H^+) and Dowex 1 (OH^-)

Two beef hearts (2–2.5 kg each) were transported in ice from the slaughter house to the laboratory, and perfused through the coronary arteries with cold distilled water. The muscular tissue was cut, ground and an acetone powder prepared from it The acetone powder (250–300 g) was suspended (1·5, w/v) in 0.4 M acetate buffer (pH 3.6) containing 0 2 M NaCl. At intervals, the suspension was vigorously stirred for a total time of 20 min by means of an Ultra-Turrax homogenizer, at room temperature. After centrifugation (15 000 × *g*, 20 min) the sediments were re-extracted as above and the supernatants were combined (With this procedure 50–85% of the enzyme activity originally present in heart muscle was recovered in this crude extract.)

The pH of the crude extract was adjusted to neutrality by addition of 2 M ammonia and ammonium sulfate was dissolved therein to make the solution 35% saturated. After 1 h the mixture was centrifuged (25 000 × *g*, 20 min), the sediments were washed once with a 35% satd solution of ammonium sulfate (pH 7) and discarded To the combined supernatant and washing solution, ammonium sulfate was added up to 45% satn After about 16 h, the precipitate was collected by centrifugation, dissolved in 0.05 M acetate buffer (pH 5 8) and desalted by filtration through a Sephadex G-25 column (3 cm × 70 cm), equilibrated with the same buffer. The red colored effluent was then subjected to ion exchange chromatography on CM-Sephadex G-50 (4 4 cm × 62 cm), previously equilibrated with 0.05 M sodium acetate buffer (pH 5 8). The column was developed with the same buffer until no material absorbing at 280 mμ was present in the effluent The first eluate was disregarded Elution was then continued with 0.15 M NaCl in 0.05 M acetate buffer (pH 5.8) and two additional protein components were collected Approx. 75% of the enzyme in the load was found to be associated with the first peak

TABLE I

PURIFICATION OF BEEF HEART β-GLUCURONIDASE

A unit of enzyme activity is defined as the amount required to catalyze the liberation of 1 μmole of phenolphtalein per min, under standard conditions

Description of step	*Vol. (ml)*	*Enzyme units*	*Protein (mg)*	*Specific activity (munits/mg)*
Heart homogenate*	—	13 1	—	0 057
1 Crude extract	2050	10 8	28 495	0 38
2 35–45% satd $(NH_4)_2SO_4$	220	7 3	1 452	5 0
3. CM-Sephadex	220	5 0	224	22 4
4 First Sephadex G-200	70	1 98	11 9	167 2
5 Second Sephadex G-200	56	0 89	1 1	809 2

* An aliquot of the heart tissue was homogenized in distilled water for 5 min by means of an Ultra-Turrax homogenizer, 0 2% (v/v) of Triton X-100 was added and the activity of β-glucuronidase determined as described Protein concentration in the homogenate was measured according to the method of GORNALL, BARDAWILL AND DAVID[8], using bovine serum albumin as standard

The fractions containing the enzyme were pooled, neutralized and concentrated by precipitation with ammonium sulfate up to 60% satn. After about 8 h, the sedimented proteins were collected by centrifugation (25 000 $\times$ *g*, 20 min), dissolved in a small volume of 0 05 M Tris–acetate buffer (pH 7) and applied to a column of Sephadex G-200 (3 cm $\times$ 120 cm), equilibrated with the same buffer. Elution was performed with 0.05 M Tris–acetate buffer (pH 7) The largest part of the fractions containing β-glucuronidase emerged just ahead of the bulk of the protein. Fractions with highest specific activity were pooled, concentrated by precipitation from 60% satd ammonium

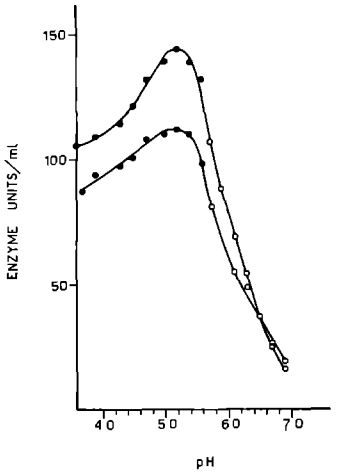

Fig 1 Effect of pH on beef heart β-glucuronidase activity The buffers used were 0 1 M acetate (pH 3 6–5 6, ●) and 0 04 M phosphate (pH 5 7–6 9, ○) Enough NaCl was added to bring the formal ionic strength to 0 16 and the final pH was recorded Upper curve 0 1% serum albumin was added to the assay medium

sulfate solution and rechromatographed on the same column of Sephadex G-200 The results of the purification procedure are summarized in Table I

Polyacrylamide gel (7%) electrophoresis of the purified enzyme was run in a refrigerator at 5 mA/gel, for 90 min, the pH of the electrode buffer being either 8 3 (ref 9) or 4.8 (ref. 10) Standard staining of the protein was carried out with Amido black[9]. The enzyme was localized in the gel by incubating an unstained column, run in parallel, under standard conditions for enzyme activity By adding the glycine–NaOH buffer, a red-violet band developed just where the substrate was split At pH 8 3 the protein migrates as a single band, in the pH 4.8 system, however, two protein bands are visible, only one revealing enzyme activity. This result obviously could reflect an insufficient purification of the enzyme, but a partial enzyme denaturation occurring during the purification and/or analytical process cannot be ruled out[11]

As shown in Fig 1, the purified β-glucuronidase exhibits a single, broad activity *versus* pH curve with a maximum at pH 5 2, which is characteristic of several mammalian β-glucuronidases[3] A second pH optimum was not observed, even on varying the substrate concentration and type of buffer (acetate (0 1 M) and piperazine–HCl (0.085 M), ionic strength 0 34) Albumin does not change the pH optimum It is interesting to note that 0 1 M citrate or phosphate buffer exerts a certain inhibitory effect on the enzyme, the inhibition being more pronounced at the lowest pH values. This result may be explained as due to a competition between these anions and the carboxyl group of the glucuronide for the enzyme[12]

Enzyme assays at different temperatures revealed that there is a 15% increase in activity per degree until 55°, and inactivation of the enzyme above this temperature An energy of activation of 14 600 cal/mole has been calculated for the enzyme reaction The purified β-glucuronidase loses over 70% of its activity when lyophilized or frozen and thawed At 4° it can be stored at least 2 weeks in 0 05 M Tris–acetate buffer (pH 7) without significant loss of activity.

The molecular weight of the purified enzyme was estimated according to the method of MARTIN AND AMES[13], using human hemoglobin, yeast alcohol dehydrogenase and beef liver catalase as reference proteins Very homogeneous data were obtained, the molecular weight of β-glucuronidase falling in the range 287 000–292 000. A Michaelis constant of $7\ 5\ 10^{-5}$ M was graphically calculated over a range of substrate concentrations from 0 05 to 0.4 mM At higher concentrations the enzyme is inhibited

The effect of several heavy metal ions on the purified β-glucuronidase has also been investigated Mn^{2+}, Ni^{2+}, and Zn^{2+}, added to the reaction medium at 1 mM concentration, do not affect the enzyme activity On the contrary 50% of inhibition was obtained with 1 mM Cu^{2+}, 0 16 mM Ag^{+} and 0 02 mM Hg^{2+} Addition of 100 μg of albumin to the assay media, so that the enzyme (about 1 μg) becomes a negligible fraction of the total protein, does not remove the Cu^{2+}, Ag^{+} and Hg^{2+} inhibition. *p*-Chloromercuribenzoate also affects the enzyme activity, producing a 50% inhibition at 0 2 mM concentration β-Glucuronidase from other tissues is also inhibited by low concentrations of Hg^{2+}, Cu^{2+} and Ag^{+}[6,12] It is generally accepted that heavy metal ions and organic mercurials are reactive towards sulphydryl groups, although the possibility of reaction with other groups should not be ignored[14]

Both neutral and amphipathic lipids inhibit the purified β-glucuronidase at very low concentrations. 0.2 mM cholesterol, 0 1 mM β-carotene (in 5% ethanol) and 10 μg/ml of Asolectin, solubilized by sonication, inhibit 86%, 53% and 98% of enzyme

activity, respectively (about 1 μg of enzyme per ml) 0.05% albumin reduces this inhibition to 74%, 32%, and 60%. TAPPEL AND DILLARD[15] have claimed that the effect of cholesterol (and retinol) on β-glucuronidase is due to disaggregation of the enzyme molecule into inactive units. This conclusion was also drawn on the basis of the experiments with albumin, known as "activator-associator", which partially prevents the inhibition.

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The use of naphthyl esters as substrates in esterase determinations

One of the most frequently used substrates for esterase determinations is α-naphthyl acetate, particularly useful in histochemical identification and in detecting esterase activity after resolution by electrophoresis or chromatography (gel, paper, *etc*)[1–3]. Recently, a study on the isoenzyme status of esterases in certain vertebrate tissues was based on the use of this ester and some related α- and β-naphthyl derivatives[4,5]

The choice of substrate in assaying esterase activity depends on several factors, the most important ones being the technique used and the specificity of the activity measured As long as a purified preparation is known to contain only one active enzyme, the generalization may be made that any ester can be used as substrate in assaying the activity of this particular enzyme Because of the multiple forms of esterases