Demonstration and Partial Characterization of Multiple Forms of Bovine Liver β-Glucuronidase*

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ABSTRACT: Chromatography of highly purified bovine liver β-glucuronidase on diethylaminoethylcellulose gave a broad unsymmetrical peak of enzyme activity. Rechromatography of single chromatographic fractions gave sharp peaks, each with approximately the same elution position as in the original chromatogram. Rechromatography of five fractions together gave five peaks of enzyme activity. These experiments showed that there were at least five and probably nine or more different, but very closely related, forms of β-glucuronidase, all of which were enveloped in the one broad peak of the original chromatogram. No chromatographic or electrophoretic system was found which could separate all of the forms into discrete zones. Since ultracentrifugation of the unfractionated mixture of β-glucuronidases did not reveal polydispersity, it is probable that the forms have similar molecular weights. Analyses of samples from across the chromatographic peak revealed no significant differences between forms in amino acid composition, specific activity, substrate saturation curves, and pH–activity curves. However, small differences in carbohydrate content were detected. It appears that β-glucuronidase is microheterogeneous in the sense that its multiple forms have similar protein structures, but differ in the amounts of some material bound to the protein.

It has been suggested that mammalian β-glucuronidase (EC 3.2.1.31) occurs in (a small number of) multiple forms, although there is disagreement concerning the nature of the forms. According to Mills *et al.* (1953), these forms are distinct β-glucuronidases with differing pH optima and kinetic characteristics. However, this view (see also Smith and Mills, 1953; Fishman and Goldman, 1965) is criticized by others who suggest that the differences among these forms might be due merely to complexes of β-glucuronidase and various tissue components (*e.g.*, Levvy and Marsh, 1960). Paigen (1961) suggested that a single kind of β-glucuronidase protein is complexed into two different subcellular structures, the lysosomes and microsomes, and some support for this view is seen in other studies (DeDuve *et al.*, 1955; Sadahiro *et al.*, 1965; Fishman *et al.*, 1967).

In the present work, the existence of multiple forms of β-glucuronidase which can be partially separated by DEAE-cellulose chromatography (Moore and Lee, 1960; Sadahiro *et al.*, 1965; Wakabayashi *et al.*, 1966, 1967) is confirmed. Indeed, preparations of bovine liver β-glucuronidase (Plapp and Cole, 1966) which appear homogeneous by several criteria were found to contain many forms of the enzyme, and these forms are shown to be very similar structurally and identical in observed enzymic properties.

Experimental Methods

The general methods and the procedure for the purification of bovine liver β-glucuronidase were described previously (Plapp and Cole, 1966). One unit of enzymic activity liberates 1 μg of phenolphthalein/hr at 37° in mM phenolphthaleinglucuronide; 19,100 of these units convert 1 μmole of substrate/min. Amino acid analyses were performed according to Moore and Stein (1963). Carbohydrate content was determined directly using the phenol–sulfuric acid method (Dubois *et al.*, 1956) and related to mannose. Visking Co. dialysis tubing was washed by boiling it 30 min in a large volume of 0.2 M Na_2CO_3 (Peterson and Chiazze, 1962), rinsing in water, boiling again 1 hr in 0.07 M sodium acetate buffer (pH 5) containing 0.5 mM EDTA·Na_3, and then soaking it in three changes of water over a period of 3 days. It was stored in 95% ethanol.

Results

Demonstration of the Existence of Multiple Forms. A DEAE-cellulose chromatogram of purified beef liver β-glucuronidase is presented in Figure 1. The specific activity across the peak was 190,000 units/mg and in all respects examined so far, the material appeared to be functionally homogeneous β-glucuronidase (*i.e.*, the enzyme was free of other proteins). However, the peak in Figure 1 is obviously not symmetrical. (Unsymmetrical peaks have been observed with all

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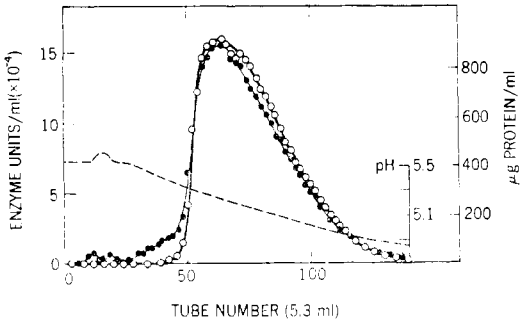

FIGURE 1: DEAE-cellulose chromatography of purified β-glucuronidase. The material was dialyzed in washed dialysis tubing against three changes of 0.05 M pyridine acetate buffer (pH 5.74) plus 0.5 mM EDTA·Na_3. It was then applied to a 1.2 × 45 cm column of 0.74 mequiv/g, 200–325 mesh DEAE-cellulose equilibrated with 0.05 M pyridine acetate buffer (pH 5.60) plus 0.5 mM EDTA·Na_3. The sample was washed in with two small aliquots of the same buffer, and starting at tube 5 the column was developed with a 720-ml linear gradient of 0.05 M pyridine (adjusted to the desired pH with acetic acid) from pH 5.60 to the limit buffer of pH 4.80 at 60 ml/hr and room temperature (23–27°). The flow rate was maintained with a Minipump. The delivery line from the gradient device to the top of the column held about 30 ml of pH 5.60 buffer initially. Each tube contained about 5.3 ml. A total of 192 mg of protein (192,000 units/mg) was applied to the column, and 93% of the enzyme and 95% of the protein were recovered in the peak, tubes 52–110. (—o—) Enzyme activity; (—●—) protein concentration; and (---------) pH of effluent.

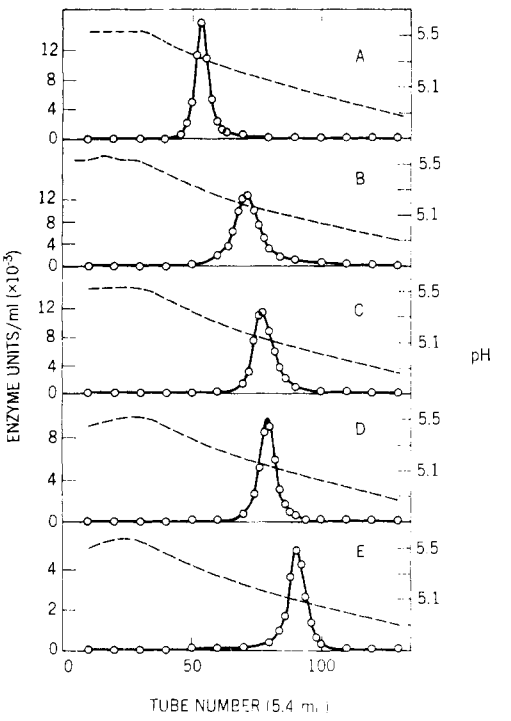

FIGURE 2: DEAE-cellulose rechromatography of fractions from the chromatogram of Figure 1. The material from a single tube was dialyzed in washed dialysis tubing against pH 5.74 buffer. It was then applied to the same column used in Figure 1 (after regeneration). The development of the column was the same as in Figure 1 except that the gradient was started at tube one. The tubes from Figure 1 which were rechromatographed were A, 54; B, 64; C, 76; D, 88; and E, 101. (—o—) Enzyme activity and (---------) pH of effluent.

preparations of bovine liver β-glucuronidase studied, whether crude or purified.) To determine whether this unsymmetrical peak was due to the chromatographic procedure or to the presence of multiple forms, samples from five selected tubes were rechromatographed individually. The results in Figure 2 show that each sample gave a very narrow peak of activity and that each rechromatographed within ±0.05 pH unit of its original position, except for tube 101 (Figure 2E) which was 0.09 pH unit earlier. This rechromatography indicated that the broad peak was not a chromatographic artifact due to transformation or to association–dissociation reactions of the enzyme during the chromatography. If such reactions were occurring one would expect the rechromatography of a single fraction to produce a broad peak similar to the one shown in Figure 1. These experiments clearly showed that the single broad peak of the original chromatogram had enveloped different forms of the enzyme which were only partially resolved by the chromatography.

It seems unlikely that conformers of the same enzyme were being separated in the first chromatography, since some days elapsed between the initial chromatography and the rechromatography. Most conformers would be expected to reequilibrate in that time. Further evidence that the multiple forms were not due to the presence of different conformers of the same molecule or to various amounts of some material loosely bound to a common protein was obtained by studying the effect of urea on an enzyme fraction. Some enzyme from tube 65 (Figure 1) was dialyzed against 7.7 M urea in 0.05 M pyridine acetate buffer (pH 5.3) containing 0.5 mM EDTA·Na_3 for 12 hr at 5°. Then the urea was dialyzed away, and the full activity of the enzyme was recovered. A DEAE-cellulose chromatogram of this urea-treated material was the same as the chromatogram of a control sample from the same fraction (a single sharp peak was obtained in both cases).

Chromatographic experiments analogous to those of Figures 1 and 2 were also performed with crude, nonautolyzed enzyme prepared from a single liver, and the results were similar. Two points were thus demonstrated: (1) β-glucuronidase from a single animal occurs in multiple forms, and (2) these forms are not simply artifacts of the purification procedure. It may be recalled that the purified enzyme was derived

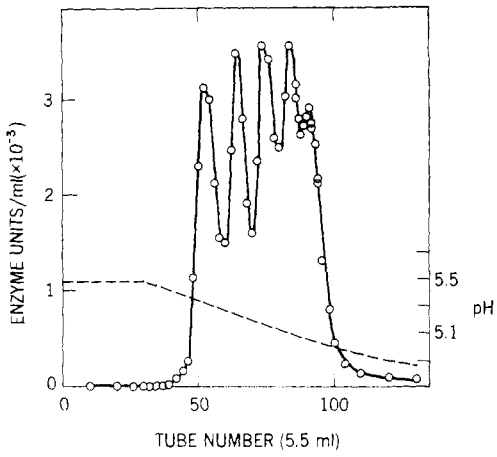

FIGURE 3: Rechromatography of mixed fractions on DEAE-cellulose. The column was developed just as the one described in Figure 1. The sample consisted of aliquots from the following tubes of the chromatogram shown in Figure 1: 53, 1.5 ml; 65, 1.0 ml; 79, 1.24 ml; 93, 2.0 ml; and 105, 3.60 ml. Of the enzyme activity applied, 97% was recovered. (—o—) Enzyme activity and (---------) pH of effluent.

from crude homogenates which were autolyzed (essentially anaerobically) at 37° for several days with limited protection against bacterial growth. Autolysis of this sort was previously shown not to alter the over-all pattern of the DEAE-cellulose chromatogram significantly (Plapp and Cole, 1966, Figure 3). Of course, changes may have occurred which affected chromatographic behavior only slightly or which produced compensating effects, but they could not be demonstrated. DEAE-cellulose chromatography of samples taken after other steps in the purification procedure, acid and alkaline ammonium sulfate fractionations, revealed some changes in the over-all chromatographic pattern, but these could very well be due to the removal of portions of particular components rather than to the alteration of any of them. Such steps are not generally considered harmful to enzyme structures. In addition, it seems unlikely that the forms were changed during the purification because the chromatographic positions, pH–activity curves, and substrate saturation curves were the same for crude and purified enzyme.

It should be mentioned that all attempts to separate the multiple forms into discrete zones by electrophoresis failed (see Plapp and Cole, 1966, for results). However, it is possible that the unsymmetrical peak obtained by zone electrophoresis at pH 5 represents a partial separation of forms (Plapp *et al.*, 1963). Chromatographic systems other than DEAE-cellulose failed to resolve the forms, although markedly asymmetrical peaks were sometimes observed (Plapp and Cole, 1966). It would thus appear that the multiple forms of β-glucuronidase are very similar.

The Number of Different Forms. When two chromatographic peaks of equal size are separated by more than the width of a peak at half-height, two peaks will be observed clearly. Knowing this, it is possible to estimate the minimum number of forms from the data of Figures 1 and 2. Since the average width at half-height observed in Figure 2 was eight tubes (and the sizes of the peaks were not very different) and since the parent chromatogram had a width of 56 tubes, there must have been a minimum of seven chromatographically distinct enzymes in the original mixture. That is, about seven peaks (of the width seen in Figure 2) would have to be added together at appropriate positions in order to reconstruct the single broad peak of the original chromatogram (Figure 1).

In order to support these conclusions, which are based on the rechromatography of single tube fractions, mixed-fraction rechromatography was performed. An equal number of enzyme units was taken from each of five positions on the chromatogram, mixed, dialyzed, and rechromatographed. As shown in Figure 3, five peaks are indeed readily distinguishable. It is quite apparent from such a chromatogram that there are more than five forms of β-glucuronidase and probably at least eight or nine forms which can be (partially) resolved by DEAE-cellulose chromatography.

To this large number of forms should perhaps be added one more. During the purification procedure, a DEAE-cellulose column is used, and some (20–30%) of the β-glucuronidase is not retained by the column (Plapp and Cole, 1966). When this material is rechromatographed, the bulk (80%) of the activity is still unretained. Therefore the lack of retention was not due simply to overloading the column. Hence the unretained peak was clearly different in its chromatographic properties from the β-glucuronidases which do adsorb to the column. (This fraction of β-glucuronidase was not studied further, since it was not obtained in pure form.)

Characterization of the Multiple Forms. As was reported in detail previously (Plapp and Cole, 1966), ultracentrifugation and Sephadex G-200 experiments failed to reveal heterogeneity in the highly purified but unfractionated mixture of β-glucuronidases (*i.e.*, material similar to that chromatographed in Figure 1). Significantly, sedimentation equilibrium experiments at low and high rotor speeds gave about the same value (within 7%) for the weight-average molecular weight, indicating that the sample was not polydisperse. It may be concluded therefore that the multiple forms which comprise the enzyme preparation have similar molecular weights of 280,000 ± 30,000.

Using material from tubes 53, 65, 79, 93, and 108 (Figure 1), enzymic properties were studied. The pH–activity curves of all of these fractions were identical, point for point, with those found previously for purified or crude enzyme (Plapp and Cole, 1966), over the pH range 3.8–8.0. The pH–activity curve in each case was broad, with a maximum at pH 4.8. Furthermore, the specific activities and the substrate saturation curves for each sample were identical.

For amino acid analyses, the samples from the rechromatography experiments (Figure 2) were rotary

TABLE I: Amino Acid Composition of Column Fractions.

Amino Acid	Moles of Residues/10^5 g of Protein[a]				
	Tube Number				
	54	64	76	88	101
Peak at 52 min[d]	2.9	0.69	Trace	5.2	12
Aspartic acid (78 min)	81	83	82	81	81
Threonine	42	43	43	44	45
Serine	47	48	47	48	51
Glutamic acid	98	97	97	98	99
Proline	48	48	50	49	52
Glycine	61	63	62	62	62
Alanine	51	50	51	51	51
Half-cystine	(4.3)	(4.1)	(6.6)	(3.5)	Trace
Valine	63	63	64	66	65
Methionine[c]	20	20	20	21	20
Isoleucine	35	35	35	36	35
Leucine	81	81	80	82	80
Tyrosine	36	46	46	19	13
Phenylalanine	41	42	42	42	41
Glucosamine	4.6	4.4	3.0	5.3	6.9
Chorotyrosine and/or tryptophan derivative (31 min)	8.0[b]	5.0	5.2	13[b]	15[b]
Lysine (43 min)	33	33	33	34	33
Histidine	27	26	27	28	27
Arginine	44	44	45	44	43

[a] Calculated by using aspartic acid, glutamic acid, and alanine as base for molar ratios (see Plapp and Cole, 1966). No correction for destruction or incomplete release of amino acids was made, since only a 24-hr hydrolysis was done. [b] The peak was skewed showing the presence of two components or more. [c] Methionine plus methionine sulfoxide. [d] Calculated using the aspartic acid color value.

evaporated to remove the pyridine acetate buffer, dissolved in 6 N hydrochloric acid, hydrolyzed, and analyzed. The results are shown in Table I. The mole ratios of amino acids agree within experimental error when the five samples are compared, except for the tyrosine values. (The amount of half-cystine is too low for reliable comparisons.) The apparent differences in tyrosine content cannot be regarded as real since these analyses indicate that tyrosine was probably being degraded during the acid hydrolysis. Note that where less tyrosine was obtained, greater amounts of material were seen at the position of chlorotyrosine and at 52 min (an unidentified peak). The degradation of tyrosine is probably due in part to the small amounts analyzed and such losses may well be aggravated by the presence of carbohydrates. It is possible that the five fractions differed in amide content, but analyses of amide levels in large proteins cannot be made with sufficient accuracy to be meaningful.

Since the enzyme appears to be a glycoprotein (Plapp and Cole, 1966), carbohydrate analyses were made on several samples from the DEAE-cellulose column As shown in Table II, small but significant differences in carbohydrate content, reported as hexose, were found. (Negligible amounts of carbohydrate were eluted from the DEAE-cellulose before or after the protein peak.) Although the over-all increase of about 25% across the peak is quite small, this would correspond to an increase in the number of residues of anhydrohexose per β-glucuronidase molecule (molecular weight of about 280,000) from 48 at the leading edge of the peak to 60 at the trailing edge. Such a difference could potentially account for as many as 13 forms of the enzyme. Of course, not only the amount but also the kind of carbohydrate present could give rise to multiple forms, and therefore even more than 13 forms might be accounted for in this way. More experiments are necessary to establish the point, but it is possible that the differences in the carbohydrate moiety are solely responsible for the multiplicity of forms. The fact that purified β-glucuronidase has less than 1 mole of phosphate/mole of protein shows that little if any

TABLE II: Analyses on DEAE-cellulose Column Fractions.[a]

Tube No.	% Hexose[b]
55	2.88 ± 0.04
61	2.84 ± 0.00
68	2.90 ± 0.05
73	3.05 ± 0.02
80	3.06 ± 0.01
85	3.18 ± 0.05
91	3.27 ± 0.06
96	3.34 ± 0.02
103	3.78 ± 0.08
109	3.57 ± 0.04

[a] These samples are fractions from the DEAE column shown in Figure 1. [b] Determined in triplicate with phenol–sulfuric acid and related to mannose (Dubois *et al.*, 1956). The figures given show the average deviations.

of the multiplicity can be due to phospholipid or nucleic acid.

Discussion

It is clear that at least nine multiple forms of bovine liver β-glucuronidase exist. From their similar chromatographic and electrophoretic behaviors, it appears that these forms are very closely related, and this is supported by the chemical, physical, and functional studies with the purified enzyme. The forms have similar molecular weights and the same amino acid compositions, pH–activity curves, and substrate saturation curves, within experimental error. The only differences observed were in carbohydrate content, differences which though small were large enough to account for at least 13 forms. Of course, it remains to be established whether or not the carbohydrate differences are the only cause of the multiplicity. (Indeed, rigorous proof that the carbohydrate is covalently bound to the protein is still lacking.) Thus, although differences in amino acid sequence have not been ruled out, it appears that bovine liver β-glucuronidase is microheterogeneous in the sense that the multiple forms have similar protein structures differing slightly in carbohydrate content or perhaps in the content of amide groups or some other still undetected material.

The chromatographic experiments reported in this paper make it extremely unlikely that the multiple forms are artifacts of the chromatography itself or of the purification procedure. It is indeed difficult to imagine that the purification procedure could alter the chromatographic properties of individual molecular forms without noticeably affecting enzymic properties or chemical properties such as molecular weight or amino acid composition.

The finding that the multiple forms have the same enzymic properties is in apparent disagreement with the results obtained by Smith and Mills (1953) from fresh or relatively fresh beef liver enzyme. Although it is true that our purified forms had been "aged" more than 3 weeks, and so might have been expected to show only the single, broad pH optimum described by Smith and Mills (1953) for their aged preparations, our experiments with fresh, crude homogenates presented pH–activity curves with exactly the same appearance as those of the purified fractions. In making such comparisons the constant ionic strength which was used in the present work may have been important since the enzyme is activated by salts (Oshima, 1936). In any case, the results were consistent with the conclusions of Paigen (1961) and Sadahiro *et al.* (1965) since the multiple forms of β-glucuronidase which they observed in crude preparations of rodent liver had the same pH–activity curves and substrate saturation curves. Since no differences in enzymic properties between forms have been found, there is no evidence that the multiple forms of β-glucuronidase have different physiological functions.

The biological significance of the multiple forms of β-glucuronidase is still unknown. Presumably all of these forms come from the lysosome since 80% of the rat liver β-glucuronidase is lysosomal (DeDuve *et al.*, 1955), and most of the enzymic activity from bovine liver was recovered in the main DEAE-cellulose peak. It is pertinent, perhaps, that other acid hydrolases from the lysosomes also have multiple forms: cathepsin D (Press *et al.*, 1960), β-galactosidase (Furth and Robinson, 1965), and ribonuclease (Maver *et al.*, 1959), arylsulfatase (Roy, 1958), and phosphatase (Moore and Angeletti, 1961). Little is known about the nature of these multiple forms, but it is possible that such multiplicity is a peculiar characteristic of lysosomal enzymes. Perhaps the structure of the lysosome itself is responsible for the multiple forms. It is thought that lysosomes have a lipoprotein membrane surrounding the enzymes (Beaufay and DeDuve, 1959), or that lysosomes are polyanionic glycolipoprotein granules with the hydrolytic enzymes retained in a latent state with ionic bonds (Koenig and Jibril, 1962). When the lysosome is ruptured, vestiges of its structure might remain bound to the enzymes.

Another possibility is that lysosomal enzymes may have bound carbohydrates or fatty acids or other material to protect them from catheptic action and that the multiplicity of forms represents various amounts of protective material bound to the proteins. (Lysosomal enzymes are quite stable during autolysis as compared to other enzymes (DeDuve and Beaufay, 1959)). The difference in amounts of carbohydrate could be the result of a relatively nonspecific glycoprotein-synthesizing system or the result of slow degradation by glycosidases during the existence of the lysosome. Finally, then, while it does not seem likely that these multiple forms of β-glucuronidase have different physiological functions, their occurrence may be a clue to the *in vivo* state of the enzyme.

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Crystallization and Properties of α-Amylase from Five Strains of *Bacillus amyloliquefaciens**

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ABSTRACT: A method for the crystallization of the α-amylase (α-1,4-glucan:4-glucanohydrolase, EC 3.2.1.1) of five strains of *Bacillus amyloliquefaciens* is described. The enzymes were found to have optimal activity and stability at pH 5.9 and 25°. The enzymes were not significantly different with respect to their electrophoretic mobility in polyacrylamide gel (pH 8.6), pH optimum, ultraviolet absorption spectrum, and immunological properties. Quantitative but not qualitative differences were noted in their activity on starch, amylopectin, glycogen, and amylose. Differences were found among the five enzymes with respect to pH and temperature stability and K_m and energy of activation values.

The α-amylase (α-1,4-glucan:4-glucanohydrolase, EC 3.2.1.1) of *Bacillus subtilis* has been studied extensively by several investigators (see review of Fischer and Stein, 1960). Owing to the diversity of strains of the organism and the experimental conditions employed by different investigators the literature on this subject is very confused. Welker and Campbell (1967a) have shown that the highly amylolytic strains of *B. subtilis* used by most investigators for the production of α-amylase are not genetically related to *B. subtilis* but are strains of *Bacillus amyloliquefaciens* (Fukumoto, 1943). We have also demonstrated that the α-amylase of authentic strains of *B. subtilis* differs from that of *B. amyloliquefaciens* in its electrophoretic and immunological properties (Welker and Campbell, 1967b).

This report is concerned with the purification, crystallization, and comparison of some general properties of the α-amylase from five strains of *B. amyloliquefaciens*.

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