Physical and Chemical Properties of β-Glucuronidase from the Preputial Gland of the Female Rat*

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In order to obtain sufficient quantities of β-glucuronidase for use in structural studies, the enzyme was purified from its richest known source, the female rat preputial gland, by a method similar to that of Ohtsuka and Wakabayashi (1969) (Enzymologia 12, 109). The purified enzyme has an $s_{20,w}^0$ of 12.5 S and a $D_{20,w}$ of $4.3 \times 10^{-13}$ cm$^2$ s$^{-1}$. Sedimentation diffusion and sedimentation equilibrium yielded molecular weights of 287,000 and 283,000, respectively. The limiting viscosity (3.6 ml/g) and the $f/f_s$ (1.08 at $\delta = 0.2$ g of H$_2$O/g of protein) indicate that the enzyme is a typical globular protein possessing little asymmetry. The circular dichroism spectrum indicates approximately 14% α-helix and a far greater amount of random coil than β structure. The enzyme is acidic, having an isoelectric point of 6.15. In electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate the enzyme exhibits a single band at molecular weight 79,000, a result indicating that the enzyme consists of four subunits of similar molecular weight. Tryptic peptide mapping suggests that the subunits are identical.

Recent studies in our laboratory have been concerned with the purification and characterization of β-glucuronidase (1, 2). The enzyme from rat liver lysosomes was purified to apparent homogeneity and was found to have a molecular weight of approximately 280,000 and to be composed of four subunits of similar or identical molecular weight (2). It contains carbohydrate, as had previously been demonstrated for the partially purified bovine liver enzyme (3). Although sufficient liver enzyme was available for kinetic and chemical modification experiments which allowed the formulation of a plausible scheme for the mechanism of action of the enzyme (4, 5), structural and other studies have been severely limited by the tedious preparation and low yield of the enzyme. For this reason, we have prepared the enzyme from the preputial gland of the female rat, which is known to be an excellent source of β-glucuronidase (6). The present paper provides physical and chemical data contributing to the understanding of the molecular structure of this enzyme.

EXPERIMENTAL PROCEDURE

Materials

Female Wistar rats (300 to 400 g) were from the exbreeder stock of Harlan Industries, Cumberland, Indiana. Exbreeder rats are normally sold at one half the cost of virgins rats. Chemicals were obtained from the following sources: bovine serum albumin, sucrose, sodium dodecyl sulfate (Catalog No. 1834), and ammonium sulfate (ultra pure), Mann; Sephadex G-50 and G-200, Pharmacia; DEAE-cellulose (DE52) and Whatman No. 3MM chromatography paper, Reeve Angel; TPCK-trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin), Worthington; phenolphthalein glucuronide and Tris, Sigma; and ampholytes, LKB. All other chemicals were reagent grade from various sources.

Methods

Buffers—The standard pH 7.5 buffer used in all experiments (unless otherwise stated) was 5 mM Tris-Cl and 0.1 M NaCl, pH 7.5.

Enzyme Assay—β-Glucuronidase was assayed in the presence of 0.1% bovine serum albumin according to Stahl and Touster (2). One unit of β-glucuronidase is that amount which catalyzes the release of 1 pmol of phenolphthalein/hour at 37° from phenolphthalein glucuronide.

Protein Concentration—During enzyme purification protein was determined by the method of Miller (7) using crystalline bovine serum albumin as a standard. Protein from column effluents was monitored by the absorbance at 280 nm. An extinction coefficient of $E_{1%}^{1cm}$ of 19 was found for purified β-glucuronidase in the standard pH 7.5 buffer. This value was based on protein concentration determined by quantitative amino acid analysis using norleucine as an internal standard.

Electrophoresis—Polyacrylamide gel electrophoresis was performed using the pH 9.5 system of Davis (8) and the pH 4.3 system of Reisfeld et al. (9). Gel electrophoresis in sodium dodecyl sulfate was carried out according to Shapiro et al. (10). Electrophoresis was conducted in a Canaco six-place apparatus equipped with a constant amperage power supply. Gels were stained for protein according to Chrambach et al. (11).

Paper electrophoresis was achieved in a Michl-type apparatus using Varsol (Esso) as the coolant.

Enzyme Purification—β-Glucuronidase was purified from the female rat preputial gland using a method slightly modified from that of Ohtsuka and Wakabayashi (12). It is to be noted that, with one exception, the modifications were minor and were made only for the sake of convenience or efficiency. The exception was the addition of a final step to remove a low s contaminant. A typical procedure is as follows: 50 rats weighing 300 to 400 g were killed and their preputial glands (8 to 10 g total) quickly removed and chilled over ice. The tissue...
was homogenized in a 6-fold volume (per weight of gland) of 0.1 M acetate buffer, pH 4.5, in a Sorvall Omni-Mixer with the homogenizing chamber submerged in crushed ice. Homogenization was done at full speed for 30 s, followed by cooling for 1 min, and then homogenization again for another 30 s. The following steps were carried out between 0 and 4°C. The homogenate was centrifuged at 15,000 rpm (27,000 × g) for 10 min in the SS-34 rotor of a Sorvall SS-3 centrifuge. The supernatant solution was saved and the pellet rehomogenized in 4 volumes of 0.1 M acetate, pH 4.5, in the same manner as before. This homogenate and the original supernatant solution were centrifuged in the SS-34 rotor at 15,000 rpm for 30 min. The resulting two supernatant solutions, containing more than 95% of the enzyme activity, were combined and treated with solid ammonium sulfate over a period of 1 min (with stirring) to 70% saturation. After stirring for 20 min at 0°, the concentration was centrifuged for 10 min at 15,000 rpm in the SS-34 rotor. The precipitate, containing approximately 90% of the original enzyme activity, was dissolved in 25 ml of 5 mM Tris-Cl, 5 mM NaCl, pH 7.5, and applied to one of two Sephadex G-200 columns (2.5 × 100 cm) (Pharmacia) connected in tandem using the upward flow technique. The elution buffer was 5 mM Tris-Cl and 5 mM NaCl, pH 7.5. The flow rate was 8 ml/hour. The enzyme routinely emerged as a symmetrical peak at approximately 480 ml. The active fraction was applied directly to a column (1 × 15 cm) of Whatman DE52 equilibrated with 5 mM Tris-Cl and 5 mM NaCl, pH 7.5, and pH 7.5, and applied to one of two Sephadex G-200 columns (2.5 × 100 cm) (Pharmacia) connected in tandem using the upward flow technique. The elution buffer was 5 mM Tris-Cl and 5 mM NaCl, pH 7.5. The flow rate was 8 ml/hour. The enzyme routinely emerged as a symmetrical peak at approximately 480 ml. The active fraction was applied directly to a column (1 × 15 cm) of Whatman DE52 equilibrated with 5 mM Tris-Cl and 5 mM NaCl, pH 7.5. The column was developed with a 500-ml linear gradient of 5 to 200 mM NaCl. The flow rate was 20 ml/hour. The enzyme eluted as a sharp but tailing peak at approximately 0.09 M NaCl. Tubes containing enzyme of specific activity greater than 2,200 units/ml, representing the bulk of the activity, were pooled and concentrated to 9 ml by an Amicon ultrafiltration apparatus with a UM-50 membrane. Three milliliters of enzyme were layered onto a 27-ml 5 to 20% continuous sucrose gradient made up in the standard pH 7.5 buffer in a cellulose nitrate centrifuge tube (25 × 75 mm) (Beckman). The tubes were centrifuged in a rotor for 45 hours at 6000 rpm, 20°, and pH 7.5 buffer. Plots of A280/A230 versus time were linear for all concentrations studied.

**RESULTS**

**Purity of Enzyme**—The purification procedure is summarized in Table I. The final yield of enzyme was generally around 50%, and the specific activity ranged from 2200 to 2300 units/mg. A comparable value was obtained by Ohtsuka and Wakabayashi (12) for their preparation of preputial gland β-glucuronidase. Following DEAE-cellulose chromatography, analytical ultracentrifugation revealed the presence of a 280-nm absorbing, low s contaminant (Fig. 1a) in our preparation. The contaminant, representing approximately 2% of the treated trypsin) for 5 hours at 37°, during which time the suspension became clear. After this first incubation period, 10 μg more trypsin were added and incubation continued for 5 hours, followed by an additional 10 μg and continued incubation for a total time of 20 hours. The sample was then lyophilized, dissolved in 0.1 ml of 0.2 M NH₄HCO₃, pH 8.5, and spotted on 3MM paper. Electrophoresis was performed for 2.2 hours at 2000 volts in pyridine-acetic acid–H₂O (1:10:89, v/v), pH 3.6. Following electrophoresis, the paper was air-dried, turned through 90°, and subjected to descending chromatography for 17 hours in butanol-acetic acid–H₂O (4:1:5, v/v, upper layer). The paper was stained with 0.2% ninhydrin in acetone, 1% in pyridine, and developed by heating for 5 min at 60°.

**Isoelectric Focusing**—Isoelectric focusing was carried out on a 220-ml LKB electrofocusing apparatus according to the instruction manual of the manufacturer. A pH 5 to 8 gradient, composed of 1% ampholines, was induced with a starting power of approximately 2 watts, and focusing was continued for 72 hours.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total units</th>
<th>Specific activity (units/mg)</th>
<th>Recovery</th>
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| Crude extract | 556 | 120,000 | 215 | 100%
| 70% (NH₄)₂SO₄ | 276 | 105,000 | 390 | 87%
| Sephadex G-200 | 27 | 57,000 | 2,300 | 52%
| DEAE-cellulose (DE52) | 25 | 57,000 | 2,300 | 48%

**FIG. 1. Analytical ultracentrifugation of β-glucuronidase before and after sucrose gradient centrifugation.** Enzyme (5.5 mg/ml) in standard pH 7.5 buffer was centrifuged at 56,000 rpm in a Beckman model E analytical ultracentrifuge at 20°. Direction of sedimentation: right to left. a. sample before sucrose gradient centrifugation; b. after sucrose gradient centrifugation.
ments were carried out. Fig. 4 shows a plot of the natural logarithm of the reduced kinematic viscosity versus enzyme concentration. Extrapolation to infinite dilution yielded a value of 3.6 ml g⁻¹, when corrected to the limiting viscosity number according to Tanford (20).

Circular Dichroism—Chen et al. (21) have recently shown that reasonable estimates of the secondary structure of proteins can be obtained by employing data derived from proteins of known conformation. Using the data given by Chen et al. (21), the best fit to the observed circular dichroism spectrum of

![Fig. 3. Polyacrylamide gel electrophoresis of β-glucuronidase. Left to right: 30 μg of purified enzyme were subjected to electrophoresis at pH 4.3 according to Reisfeld et al. (9), 25 μg of purified enzyme run at pH 8.9 according to Davis (8), and 25 μg of purified enzyme run at pH 7.1 in sodium dodecyl sulfate according to Shapiro et al. (10).](image)

![Fig. 2. Sedimentation equilibrium of β-glucuronidase. Run performed at 13,000 rpm at 5°C in the AN-D rotor. Enzyme concentration was 0.4 mg/ml in the standard pH 7.5 buffer. Protein concentration was measured using interference optics.](image)

![Fig. 4. Determination of limiting viscosity number of β-glucuronidase. Viscosity measurements were carried out at 20.0 ± 0.01°C in a 2 ml capacity Cannon-Ubbelhode dilution viscometer. The data are plotted according to Kragh (19). For details see “Methods.”](image)
β-glucuronidase (Fig. 5) was calculated by computer program to be 14% α helix, 4% β structure, and 81% random coil.

**Sodium Dodecyl Sulfate Gel Electrophoresis**—The subunit molecular weight of preputial gland β-glucuronidase was determined using sodium dodecyl sulfate gel electrophoresis. As shown in Fig. 6, β-glucuronidase migrated to a position corresponding to a molecular weight of 72,000. Inasmuch as only one protein band was detected, the enzyme is apparently composed of four subunits of similar or identical molecular weight.

**Tryptic Maps**—To ascertain whether the subunits were identical with respect to amino acid sequence, the enzyme was digested with trypsin and the peptides subjected to mapping. As shown in Fig. 7, the tryptic digest yielded 57 ninhydrin-positive spots out of a theoretical 61, indicating that the subunits are very similar, if not identical, in amino acid sequence.

![Figure 5](image1.png)

**Fig. 5.** Far ultraviolet circular dichroism spectrum of β-glucuronidase. Experiment performed using a Cary model 60 spectropolarimeter equipped with a 6001 circular dichroism attachment. Enzyme (0.68 mg/ml), in standard pH 7.5 buffer, was placed in a cuvette of path length 1 mm and was scanned using a full scale range of 0.1 degree at a time constant of 3.

![Figure 6](image2.png)

**Fig. 6.** Determination of subunit molecular weight of β-glucuronidase by sodium dodecyl sulfate gel electrophoresis. The experiment was performed according to Shapiro et al. (10). Molecular weight standards are: (1) bovine serum albumin dimer, (2) bovine serum albumin monomer, (3) pyruvate kinase, (4) enolase, (5) ovalbumin, (6) creatine kinase, (7) alcohol dehydrogenase, (8) glyceraldehyde 3-phosphate dehydrogenase, (9) pepsin, (10) chymotrypsinogen, and (11) lysozyme. Arrow indicates position of preputial gland β-glucuronidase.

**Isoelectric Point**—When analyzed by the method of isoelectric focusing, β-glucuronidase migrated to a position corresponding to an isoelectric point of 6.15, a value identical with that obtained by Ohtsuka and Wakabayashi (12) for their preparation of preputial gland β-glucuronidase.

**Discussion**

The isolation of sufficient quantities of β-glucuronidase necessary to carry out the structural studies reported in this paper was made possible by employing the richest known source of the enzyme, the female rat preputial gland. That the preputial gland is an ideal source of β-glucuronidase was well demonstrated by Ohtsuka and Wakabayashi (12), who used a four-step procedure to prepare 13.8 mg of β-glucuronidase from 100 virgin rats. In the procedure presented in this paper we have used larger (and less expensive) exbreeder rats and consequently nearly quadrupled the yield per rat while halving the cost. The specific activity of our preparation of preputial gland β-glucuronidase is very similar to the preparation of Ohtsuka and Wakabayashi (12). However, these authors report that incubation in the cold increases the specific activity by almost 50%, a phenomenon which they attribute to renaturation. We have been unable to confirm this activation procedure and have sought a plausible explanation. One possibility, that the added sucrose gradient centrifugation step removes a substance required for the activation, was ruled out when it was shown that the post DEAE material could not be activated. Another possibility was that the enzyme from exbreeder rats could not activate. However, when the enzyme was isolated from virgin rats, the product obtained exhibited the same specific activity as the exbreeder preparation and, furthermore, did not activate in the cold. Two remaining possibilities, a difference in the strain of the rats used and minor modifications in the purification procedure, have yet to be ruled out. It is noteworthy that our preparation has an isoelectric point identical with the preparation of Ohtsuka and Wakabayashi.

![Figure 7](image3.png)

**Fig. 7.** Map of tryptic peptides of β-glucuronidase. Experiment was carried out on Whatman No. 3MM chromatography paper. Line indicates origin. For details, see "Methods."
gland β-glucuronidase is very similar to rat liver lysosomal enzyme. Hence, we have carried out compositional (16) and spectral (26) studies on the preputial gland enzyme.

Acknowledgments—We are grateful to Dr. C.-C. Wang for frequent advice during the course of this study, and we are indebted to Dr. Howard Six for assistance with ultracentrifugation studies.

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