Microheterogeneity of *N*-Acetyl-β-D-hexosaminidase of Bull Epididymis[1] (37047)

KIYOSHI HAYASE, SAMUEL R. REISHER, AND DAVID KRITCHEVSKY

Harrison Department of Surgical Research, University of Pennsylvania School of Medicine and The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

In human tissues, *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.30) is present in two major modifications, namely the A and B forms (1–3). It has also been demonstrated that epididymis is the richest source of this enzyme (4). The *N*-acetyl-β-D-hexosaminidase of pig epididymis has been partially purified (4), and shown by ion exchange chromatography to exist in two forms (5). By use of isoelectric focusing, Winchester (6) recently demonstrated the separation of several forms of the enzyme from ram epididymis. Since some deficiency of the enzyme appears to be associated with certain diseases (2, 7), elucidation of the molecular species of the enzyme was considered of interest.

The present report deals with the demonstration by gel electrophoresis of multiple forms of *N*-acetyl-β-D-hexosaminidase in bull epididymis in comparison with the two forms present in human aorta (8). We have utilized the direct coupling reaction of naphthol with diazotized dye (9) to localize the enzyme on the gel.

Materials and Methods. Bull epididymis was obtained fresh, cleaned and either used immediately, or stored at −20° until use. No change in number and pattern of electrophoretically separable *N*-acetyl-β-D-hexosaminidases was found between fresh epididymis and samples frozen for as long as 5 mo.

Epididymis was homogenized in 10 vol (w/v) of 0.05 *M* citrate buffer (pH 4.3) using a glass–glass homogenizer. The homogenate was centrifuged at 16,000*g* for 60 min and the resultant clear supernatant was used for further study. Recovery of *N*-acetyl-β-D-hexosaminidase activity was approximately 90% of that found in the homogenate. When deionized water was used for homogenization instead of buffer, the release of enzymic activity was found to be less than 35%. Preparation of human aortic extract has been described elsewhere (8).

Routine assay of total enzyme activity was carried out as previously described (10), employing *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate.

Polyacrylamide gel electrophoresis was performed according to the technique of Smith (11) and Hjerten, Jerstedt and Tiselius (12) utilizing a continuous buffer system at 5.3% gel concentration. A mixture of acrylamide solution (20 g of monomer and 1.2 g of bis/100 ml H_2O), 0.377 *M* Tris–glycine buffer (pH 9.5) with 0.12% of *N,N,N′,N′*-tetramethylethylenediamine and ammonium persulfate solution (0.1 g/100 ml H_2O) at a ratio of 1:1:2 (v/v/v) was deaerated, poured into glass tubes (0.5 × 7.5 cm), and allowed to polymerize at room temperature. After polymerization, the gel tubes were kept at room temperature for at least 3 hr before use.

Samples (5–50 μl) were layered on top of the gel (cathodic side) with concentrated sucrose solution. Both upper and lower reservoir buffer was 0.094 *M* Tris–glycine (pH 9.5). Electrophoresis was carried out in an apparatus which accommodates 8 such tubes simultaneously. Human serum (5 μl) with bromphenol blue (BPB) was added to one of the tubes to routinely check the migration and separation of proteins. Application of 2.5 mA/tube at 4° resulted in the migration

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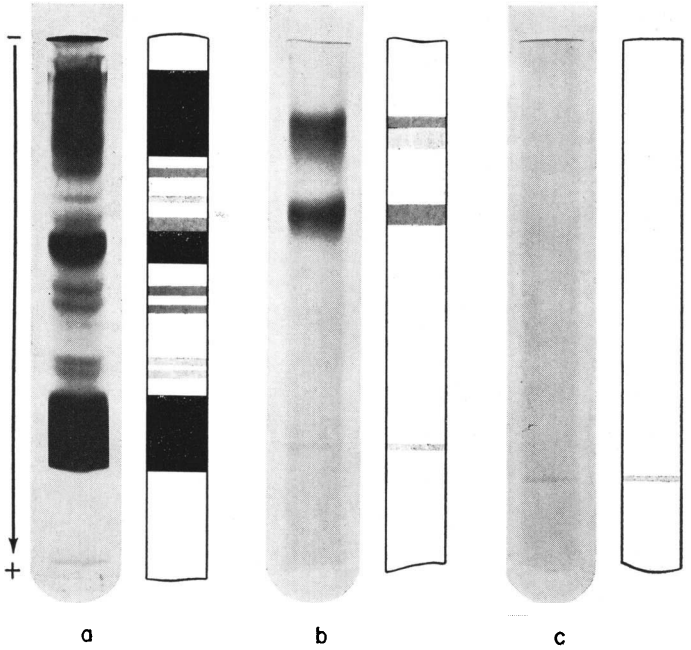

FIG. 1. Electrophoresis of human serum and *N*-acetyl-β-D-hexosaminidase of human aorta. Conditions: gels were prepared at 5.3% and electrophoresis was performed at 2.5 mA/tube for 30–40 min at 4°. The details are described under Materials and Methods. (a) protein staining of human serum with 0.1% amido black; (b) enzyme staining of human aorta—the A form is the fast-moving band (lower band in the gel) and the B form is the upper band. The sharp, faint band near the end of the gel in (b) and (c) was due to the staining of albumin with the fast garnet GBC; (c) the same as (b), except that *N*-acetyl-glucosaminolactone (1 m*M*) was added in the incubation mixture.

of BPB through the entire length of the gel in 30–40 min.

After electrophoresis, gels were removed from the tubes; protein was stained with 0.1% amido black in 7% acetic acid for 1 hr at room temperature, then destained electrolytically. *N*-Acetyl-β-D-hexosaminidases were localized using an incubation system similar to that originally described by Hayashi (13) for histochemical demonstration of the enzyme in the tissues. The incubation mixture for each gel consisted of 1.5 mg of *N*-acetyl-β-D-glucosaminide of naphthol AS-BI (7-bromo-3-hydroxy-2-naphth-*O*-anisidide, Pierce Chemical Co., Rockford, IL) dissolved in 0.5 ml of ethylene glycol monomethyl ether, 9.5 ml of 0.1 *M* citrate buffer (pH 4.3) and 10 mg of fast garnet GBC (*O*-amino azotoluene, diazonium salt, Sigma Chemical Company, St. Louis, MO). The solution is stable for at least 3 wk when stored at 4°. Each gel was incubated at 37° for 90 min in order to obtain suitable staining (violet color) at the site of enzyme. The reaction was stopped by immersion of the gels into 7% acetic acid. For optimum results, it is important that the rate of the enzymatic reaction should not exceed the rate of the coupling reaction.

Action of neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* (Worthington Biochem. Corp., Freehold, NJ) on *N*-acetyl-β-D-hexosaminidases was followed in the incubation mixture of 0.04 ml (0.32 units) of neuraminidase (free from *N*-acetyl-β-D-hexosaminidase activity), 0.02 ml of concentrated bull epididymis preparation and 0.04 ml of 0.1 *M* acetate buffer (pH 4.6) at 37°.

An inhibitor of the enzyme, *N*-acetyl-glucosaminolactone, was synthesized by acetylation of glucosaminic acid according to Findlay, Levvy and Marsh (14). When used, an

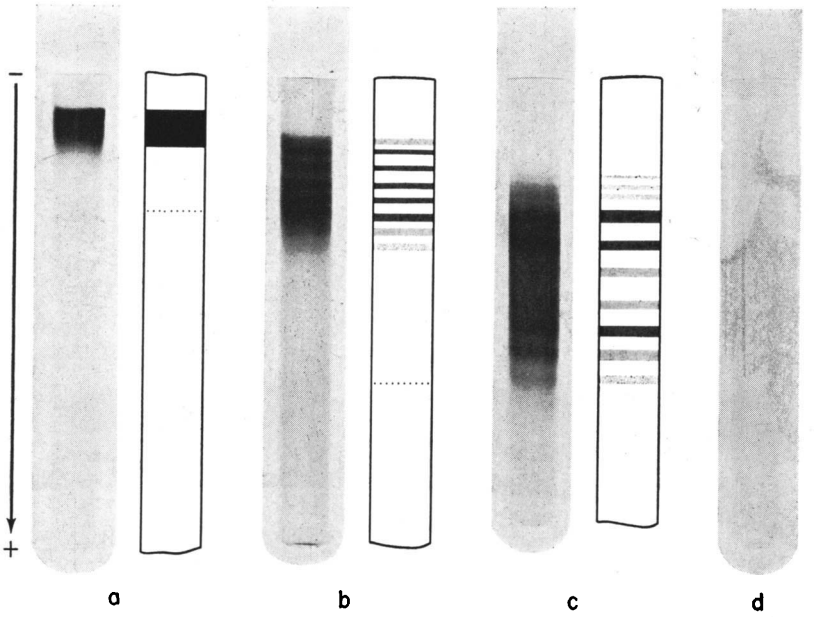

FIG. 2. Electrophoretic separation of *N*-Acetyl-β-D-hexosaminidase of bull epididymis. Conditions: Electrophoresis was carried out at 2.5 mA/tube at 4° with 10 μl of bull epididymis supernatant for (a) 30 min; (b) 60 min; (c) 90 min; (d) 90 min (*N*-acetyl-glucosaminolactone, 1 m*M*, added to the incubation mixture).

appropriate amount (1 m*M*) of the inhibitor was added to the incubation mixture for enzyme localization as described above.

Results and Discussion. Gel electrophoresis patterns are shown in Figs. 1–3. Since some bands show up faintly on the photographs, we have accompanied each photographed pattern with a schematic diagram depicting the location and intensity of each band.

The pattern of protein separation from human serum in the presently employed continuous as opposed to discontinuous buffer systems (12) is shown in Fig. 1a. With 5.3% gel concentration all *N*-acetyl-β-D-hexosaminidases from bull epididymis and human aorta migrated into the gel and were well separated.

It is shown in Fig. 1b that human aortic *N*-acetyl-β-D-hexosaminidase is clearly separated into two bands, which have been identified as the A form (fast-moving band) and the B form (slow-moving band). These bands have been characterized by ion-exchange chromatography and have been partially purified in our laboratory (8). Although further separation of two bands was not achieved, the B form appears to consist of several bands which migrate together. Otherwise, the result is in agreement with those found in other human tissues (1, 2, 7). As shown (Fig. 1c), the gel incubated with 1 m*M* *N*-acetyl-glucosaminolactone did not show any staining, indicating that the observed bands were due to the presence of *N*-acetyl-β-D-hexosaminidase. The same amount of inhibitor was also found to inhibit the enzyme activity completely when incubated with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. The faint band near the bottom of the gels (b, c) is due to albumin which stains yellow with this dye and can easily be distinguished from enzymatic bands (violet) in the original gels.

In Fig. 2, migration and separation of bull epididymis *N*-acetyl-β-D-hexosaminidase is illustrated. Gels were run at 2.5 mA/tube for 30(a), 60(b) and 90(c) min, respectively. It is noted in the 30 and 60 min gels that a small portion of the enzyme (very faint band) migrates well ahead of the major portion of enzyme. Electrophoresis for 90 min gave the best separation and as many as 12

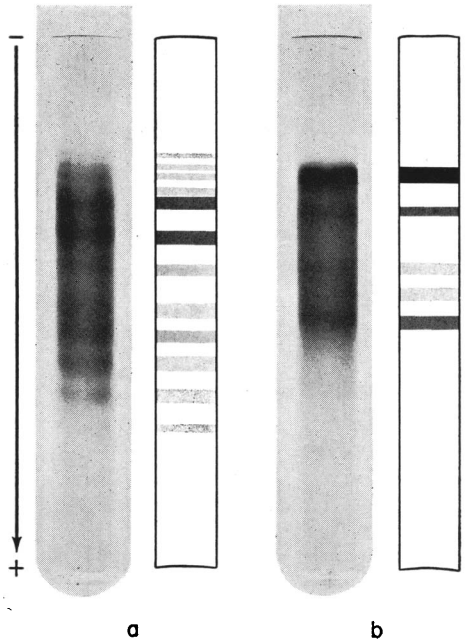

FIG. 3. Action of neuraminidase upon *N*-Acetyl-β-D-hexosaminidase of bull epididymis. Conditions: Electrophoresis at 2.5 mA/tube for 90 min at 4°. (a) bull epididymis supernatant; (b) bull epididymis supernatant incubated with 0.32 units of neuraminidase for 60 min at 37°. An equal amount of the supernatant (10 μl) was applied on both tubes.

bands were detectable.

It is interesting that Robinson and Stirling (1) estimated that the A form of the enzyme from human spleen "might consist of B form with up to 12 short carbohydrate side chains terminating with a sialic acid residue." Involvement of artifacts during preparation of the homogenate of bull epididymis is rather unlikely, since (a) human aortic extract prepared in a similar manner showed the usual two forms of the enzyme, and (b) different epididymal samples exhibited the same distribution pattern of the enzyme.

Figure 2d also demonstrates that as with human aortic enzymes (Fig. 1c) 1 m*M* *N*-acetyl-glucosaminolactone completely abolishes the appearance of bands with enzyme activity in bull epididymis preparation. In a separate experiment, 100% inhibition was confirmed by incubation with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide.

In order to prove that some forms of bull epididymal *N*-acetyl-β-D-hexosaminidases are sialoglycoproteins, the preparation was treated with neuraminidase of varying concentration and for different times. It is expected that after removal of the acidic sialic acid moiety, the corresponding asialoenzyme must migrate at considerably lower mobility in the same electrophoretic field. The results are presented in Fig. 3 which demonstrates the difference between preparations untreated (a) and treated (b) with neuraminidase for 60 min at 37°. The treatment eliminated some of the fast-moving bands and caused a slower moving band at the top of the gel to stain more deeply. After the treatment, five distinct bands at the top remained from original 12 bands with some change in mobility. These findings suggest that at least some of the fast-moving bands are sialoglycoproteins in their native state and that after removal of sialic acid, they are transformed into slower moving moieties. The fact that total *N*-acetyl-β-D-hexosaminidase activity (measured with *p*-nitrophenyl-β-D-glucosaminide) was found unchanged before and after neuraminidase treatment indicates that these various forms of enzyme act equally upon synthetic substrate (2) and is unlikely that the sialic acid moiety is involved in the active site of the enzyme.

We were unable to demonstrate with various treatments with neuraminidase, however, total transformation of various forms of the enzyme into a single slow-moving form. Robinson and Stirling (1) estimated approximately 45% of the activity of the A form of human splenic *N*-acetyl-β-D-hexosaminidase was transformed into B form by neuraminidase treatment.

Summary. Most (90%) of the total activity of *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.30) of bull epididymis homogenate was found in the soluble fraction. Electrophoresis of the soluble fraction on polyacrylamide gel (5.3%) at pH 9.5 separated the enzyme into as many as 12 enzymatically active bands. The bands were successfully stained on the gel using naphthol AS-BI *N*-acetyl-β-D-glucosaminide as substrate; freed naphthol being coupled with fast garnet GBC. Under similar conditions, human aortic *N*-acetyl-β-D-hexosaminidase was separated into two bands, namely the A and B forms.

In both preparations, 1 m*M* *N*-acetyl-glucosaminolactone completely inhibited staining.

Incubation of bull epididymal preparation with neuraminidase resulted in the disappearance of some of the fast-moving bands and the concomitant deeper staining of the slow-moving bands. Some changes in relative mobility were also observed.

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