AMPHOLYTE DISPLACEMENT CHROMATOGRAPHY - A NEW TECHNIQUE FOR THE SEPARATION OF PROTEINS ILLUSTRATED BY THE RESOLUTION OF β-N-ACETYL-D-HEXOSAMINIDASE ISOENZYMES UNRESOLVABLE BY ISOELECTRIC FOCUSING OR CONVENTIONAL ION-EXCHANGE CHROMATOGRAPHY.

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Summary

The 'B' variant of pig epididymal β-N-acetyl-D-hexosaminidase has, for the first time, been resolved into two isoenzymes (designated Bα and Bβ). Like the 'B' variant of ox liver aryl sulphatase, the two β-N-acetyl-D-hexosaminidase isoenzymes could not be resolved by isoelectric focusing procedures, but could be separated by ion-exchange chromatography. Similar difficulties may be expected in the isoelectric focusing of other large, moderately basic proteins with closely similar isoelectric points and with a tendency to aggregate. The β-N-acetyl-D-hexosaminidase 'B' isoenzymes were successfully resolved using ampholyte displacement chromatography - a new technique in which the proteins are displaced from an ion exchange adsorbent using mixtures of amphoteric substances of closely similar isoelectric points.

There is intense current interest in the multiple forms of mammalian β-N-acetyl-D-hexosaminidases, but the nature of the molecular differences between the isoenzymes is still very uncertain.[1] Separation techniques which depend upon the electric charge properties of proteins have been used extensively in the characterization of β-N-acetyl-D-hexosaminidases and the various isoenzymes have been designated 'A', 'B', 'C' etc.. Recently, the authors described[2,3] how isoelectric focusing could be used as a valuable guide in a new systematic strategy for the purification of proteins, and this was illustrated by the purification of the 'B' variant of the β-N-acetyl-D-hexosaminidase from pig epididymis. During subsequent experiments it was noted that the freshly prepared enzyme rarely, if ever, showed really sharp zones on gel or density-stabilized isoelectric focusing: it was also noted that the enzyme showed signs of further fractionation during displacement from an ion-exchange cellulose by mixtures of carrier ampholytes. These two effects

are described in the present communication and have led both to a possible explanation for difficulties in the sharp focusing of the hexosaminidase B, and to a new experimental procedure for the separation of proteins.

Materials and Methods

The pig epididymal enzyme used here was the partially purified preparation described earlier[3] and consisted of the combined active fractions from Sephadex G200 chromatography, concentrated by ultrafiltration and then reconstituted in 0.02M Tris/HC1 pH 7.6. β-N-acetyl-D-hexosaminidase activities (EC.3.2.1.30) were assayed using either the p-nitrophenyl or 4-methylumbilliferyl glycosides as described earlier.[3]

Isoelectric focusing was carried out at 5° in an L.K.B. 8101 density stabilized column using 600 volts and 1:2 mixtures of pH 7-9 and pH 8-10 L.K.B. 'Ampholine' carrier ampholytes to a total concentration of 1% (w/v). A considerable variety of other carrier ampholyte mixtures was tried but none gave better results (i.e. shallower, more even pH gradients or greater isoenzyme resolution) than the above formula.[4]

Column chromatography was carried out at 5° using columns (45mm x 11mm diam.) of CM-cellulose (CM52; Whatman Biochemicals, Maidstone, Kent, U.K.) which had been equilibrated carefully at 5° with 0.02M Tris/HC1 buffer, pH 7.6. Elution of adsorbed enzyme was carried out variously as described in the text.

Results

After density gradient stabilized isoclectric focusing for 24hr., the partially purified 'B' variant of epididymal β-N-acetyl-D-hexosaminidase typically gave elution profiles of the kind shown in Fig. 1. with a fairly broad band of activity over the range pH 7.5-9.2. Essentially the same isoelectric spectrum was obtained for the enzyme after isoelectric focusing for 72hr. or after using a higher (1000V) overall potential difference, but under these conditions the enzyme activity was spread over fewer fractions due to the contraction of the basic pH gradient as a consequence of the well-established 'plateau' effect.[5]

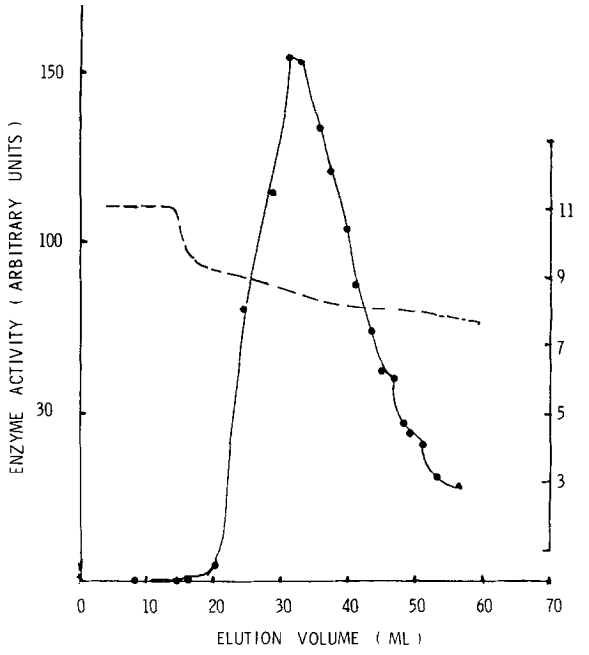

Fig. 1. β-N-acetyl-D-hexosaminidase (—●—) and pH gradient (- - -) in fractions from a density-gradient stabilized isoelectric focusing column after focusing a partially purified (i.e. molecular size fractionated) epididymal enzyme preparation.

The enzyme preparation (1ml) was adsorbed on a column of CM-cellulose at pH 7.6 and at 5° and was eluted with a 4% (w/v) mixture of pH 8-10 'Ampholine' carrier ampholytes to give the enzyme elution profile shown in Fig. 2a. Enzyme recoveries were typically 80-100% and when each of the two main peaks (designated Bα and Bβ as shown) were re-run individually on fresh columns they appeared at the same relative positions in the eluate as before.

A sample of the same enzyme preparation was adsorbed on a similar column and eluted with a gently rising concentration gradient of salt, but this failed (Fig. 2b) to resolve the enzyme into species analogous to those shown in the previous experiment (Fig. 2a). No improvement in the resolving power of this conventional ion-exchange elution procedure resulted, using even shallower salt concentration gradients or by employing a salt gradient in conjunction with a 0.02M Tris/HC1 buffer pH 8.5 (i.e. at a pH nearer the estimated mean isoelectric points of the component isoenzymes). Dialysis against 0.02M Tris/HC1 buffer

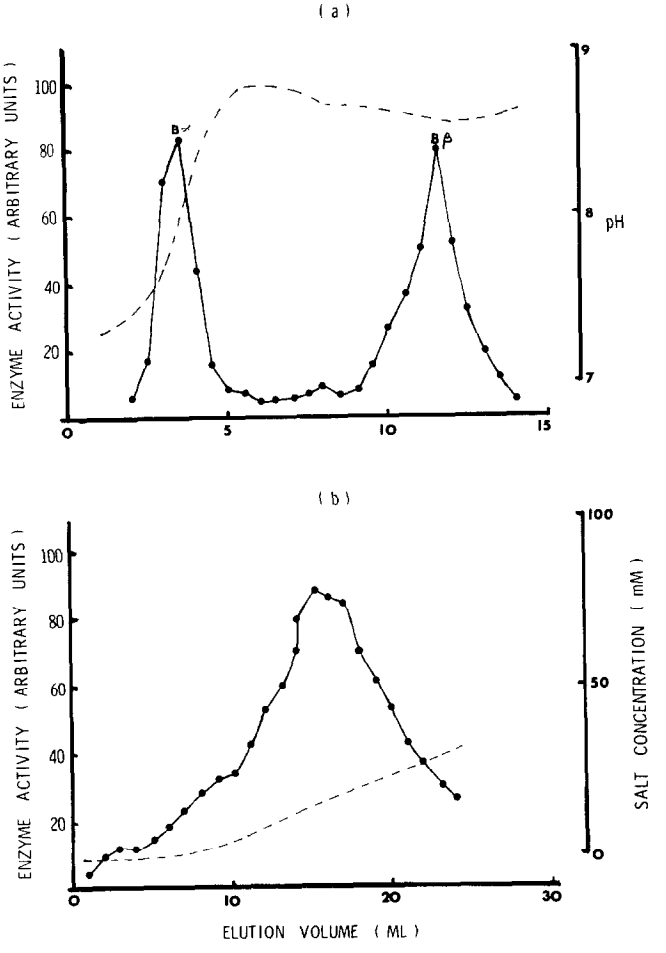

Fig. 2 (a) β-N-acetyl-D-hexosaminidase activity (●—●) and pH (--) profiles in eluant from a column (45mm x 11mm) of CM-cellulose after loading with partially purified epididymal enzyme and eluting with 4% (w/v) pH 8-10 Ampholine carrier ampholytes. (b) β-N-acetyl-D-hexosaminidase activity (●—●) and NaCl concentration (▪--▪) profiles from a column loaded as above but eluted with 0.02M Tris/HCl buffer pH 7.6 and an increasing concentration of NaCl.

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pH 7.6 of the combined active fractions from the unresolved enzyme peak shown in Fig. 2b, followed by adsorption and elution of the enzyme by the ampholyte displacement procedure showed the presence of approximately equal activities of the Bα and Bβ components.[4]

The profile of enzyme activity eluted by ampholyte displacement was very reproducible and the procedure very convenient (e.g. in not requiring a gradient

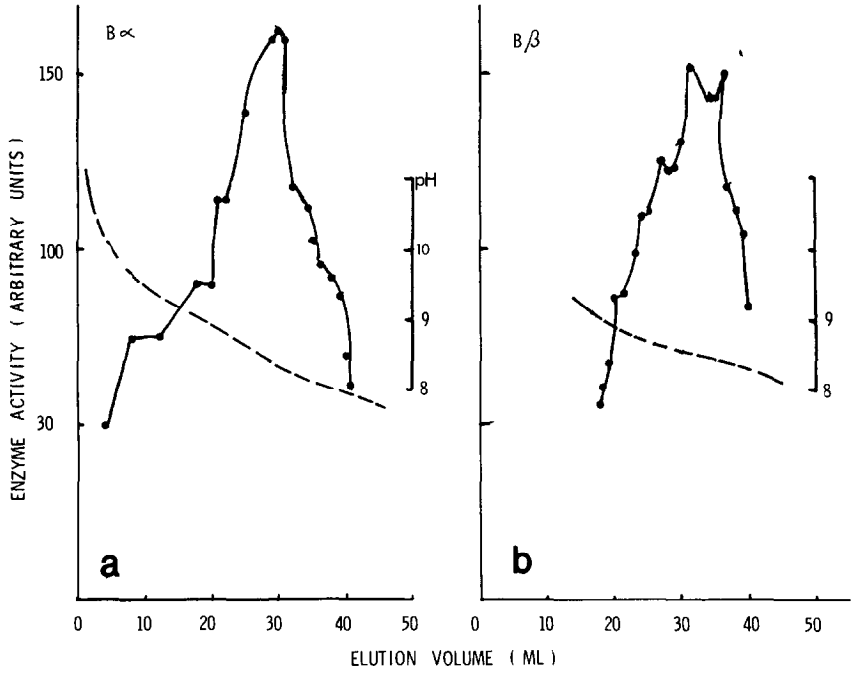

Fig. 3. β-N-acetyl-D-hexosaminidase activities ( ) and pH gradients (----) from density-gradient stabilized isoelectric focusing columns after focusing for 24hr. at 5 and 600V. a) the Bα enzyme peak illustrated in Fig. 2a, and b) of the corresponding Bβ peak.

forming device). The resultant Bα and Bβ enzyme species were then subjected separately to density stabilized isoelectric focusing and the results show - (Figs. 3a and 3b) that while the component isoenzymes gave slightly different mean isoelectric points (Bα somewhat more acidic than Bβ), the difference was insufficient to expect a discrete separation by isoelectric focusing.

Discussion

In 1968 Allen and Roy[6] reported that the 'B' variant of ox liver aryl sulphatase could be separated into two components (designated Bα and Bβ) by ion exchange chromatography. These two components showed closely similar isoelectric points in the pH region 8.2-8.3 when subjected individually to isoelectric focusing. However, the Bα and Bβ sulphatase components could not be resolved by

isoelectric focusing alone and the authors concluded "..that the fractions obtained by isoelectric focusing are aggregates of sulphatases Bα and Bβ which are stable at the low ionic strengths used in this procedure..". They went on to say that "It is not yet possible to decide whether this is a general hazard in isoelectric focusing....".

The work described here on β-N-acetyl-D-hexosaminidase isoenzymes shows some remarkable parallels with the findings of Allen and Roy[6]. Thus, the subject of both investigations was a basic 'B' variant of a lysosomal enzyme; in both cases the 'B' variant could be resolved by ion-exchange chromatography into two components which differed slightly in isoelectric point, but which could not be resolved by isoelectric focusing procedures. The principal difference between the two investigations is that the 'B' components of the hexosaminidase could not be resolved by conventional salt elution from the ion-exchange cellulose but could be displaced selectively by amphoteric ions of closely similar isoelectric points. The precise physico-chemical reason for the effectiveness of this 'ampholyte displacement chromatography' is uncertain, but it seems likely that it depends upon a very effective competition of amphoteric substances, (proteins and carrier ampholytes) for sites on the ion-exchange adsorbent. This new technique developed out of a procedure designed to 'interface' between two stages of the proposed systematic strategy for proteins purification.[3] Thus, the enzyme was adsorbed on the ion-exchange material under conditions indicated from the results of gel isoelectric focusing, and then displaced by appropriate mixtures of carrier ampholytes. In this way, the presence of undesirable buffer ions was avoided and, at the same time, conditions were established suitable for a final fractionation by density-stabilized isoelectric focusing.[3] A comparison of the procedures and results represented in Figs. 2a and 2b illustrates the effectiveness of the ampholyte displacement chromatography and that it is a fractionation technique in its own right which should find further applications.

It is concluded that the 'B' variant of pig epididymal β-N-acetyl-D-

hexosaminidase is readily separable into two principal components of similar isoelectric point using the new chromatographic technique, but that the components are not separable during isoelectric focusing due to association of the component proteins under the lower'ionic strength' conditions involved in the isoelectric focusing process.

Similar difficulties may be expected in the isoelectric focusing of other large, moderately-basic proteins of similar isoelectric point, since such proteins may well move slowly with comparable mobilities in a rapidly established pH gradient of relatively low 'ionic strength' towards a pH region where there is often difficulty in focusing proteins.[7]

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