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# HIGH-CAPACITY METHOD FOR PURIFICATION OF HUMAN LIVER HEXOSAMINIDASE B USING HYDROPHOBIC CHROMATOGRAPHY

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## SUMMARY

Hydrophobic chromatography was investigated as a purification procedure for human liver hexosaminidases. Both phenyl-Sepharose and valine-Sepharose have a high binding capacity for hexosaminidases. A degree of resolution between the A and B isozymes is achieved with phenyl-Sepharose.

Both hydrophobic supports must be used close to their capacity in order to recover the applied enzyme.

Two purification procedures for human liver hexosaminidase B were employed, which resulted in recoveries of approximately 48 and 24% with final specific activities of 33,400 and 4840 nmole/min·mg, respectively.

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## INTRODUCTION

Large amounts of homogeneous enzyme are required for many biochemical investigations. The limiting step in such studies, therefore, is often the development of high-capacity procedures for protein purification.

Many classical purification procedures cannot be adapted to the processing of the large amounts of tissue protein that must be used when the enzyme of interest constitutes only a small fraction of total cell protein. Other methods are too severe and lead to significant losses of enzyme activity in the final preparation.

One answer to the need for purification procedures with higher capacity is the development of affinity chromatography. The preparation of affinity adsorbents, however, often involves trial and error in the choice of ligands and spacers and yields purification steps that are useful for only one enzyme. A more general technique for the enhancement of enzyme purification capacity has been the use of hydrophobic chromatography[1].

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We have investigated the utility of this type of chromatography in the purification of human liver hexasaminidase B. Some of the parameters that affect the capacity, resolution and recovery of the enzyme from hydrophobic columns were investigated. These procedures were combined with solubility and ion-exchange chromatography steps to yield an enrichment of 5000-fold with a recovery of 48%.

## EXPERIMENTAL

### *Hexosaminidase activity*

Hexosaminidase was assayed fluorimetrically[2]. A unit of activity is defined as that amount of enzyme which catalyses the hydrolysis of 1 nmole of substrate per minute at 37°. Hexosaminidase B was determined in the presence of hexosaminidase A after heating the enzyme for 30 min at 54°[3]. Protein was determined by the method of Warburg and Christian[4].

### *Gel electrophoresis*

Polyacrylamide gel electrophoresis was performed according to Reisfeld *et al.*[5]. The gels were cut longitudinally and half-gels stained for protein using amido black and for hexosaminidase activity[6].

### *Preparation of human liver extract*

Human livers were obtained within 4 h post-mortem and stored at −20°. The homogenate and supernatant were prepared as described previously[7].

### *Chromatographic supports*

DEAE-Sephadex A-50 and phenyl-Sepharose were purchased from Pharmacia (Uppsala, Sweden). Valine-Sepharose was prepared according to Rimmerman and Hatfield[8]. Acid hydrolysis and automated amino acid analysis of the valine-Sepharose gave a degree of substituion equal to 10 μmole/ml of packed support.

## RESULTS

### *Use of combined solubility steps for purification of hexosaminidase B*

The resistance of hexosaminidase B and, to a lesser extent, hexosaminidase A, to denaturation in buffers of low ionic strength at acidic pH is a characteristic shared by other lysosomal enzymes but not by bulk liver proteins. The starting extract ("liver supernatant") with a protein concentration of 63 mg/ml and a specific activity of 7 units/mg of hexosaminidase was dialysed three times against 10 volumes of 2.5 m*M* sodium citrate–sodium phosphate buffer (pH 4.4) at 4° for 72 h. This procedure, used as step 2 in both procedures I and II (Table II), resulted in a recovery of 100% of hexosaminidase activity in the supernatant obtained after centrifugation of the retentate at 13,000 *g* for 30 min at 4°. Approximately one third of the liver supernatant protein remained soluble.

The clear "dialysed supernatant" obtained after centrifugation tended to become turbid on storage at either 4° or −20°, suggesting that more liver supernatant protein could be eliminated by more drastic exploitation of its limited solubility in the acidic buffer.

Purification steps I-3 and II-3 represent different approaches to the further enrichment of specific activity using procedures based on the differential solubility of hexosaminidases and bulk liver protein. In step I-3, the "dialysed supernatant" was mixed with an equal volume 0.1 *M* sodium citrate–sodium phosphate buffer (pH 4.4) and incubated for various intervals at 37°. After 3 h, precipitation of protein was complete. The supernatant obtained after centrifugation as described above was enriched 5-fold in specific activity compared with the liver supernatant.

In step II-3 the procedure used was based on complete precipitation of dialysed supernatant protein with saturated ammonium sulphate, followed by differential extraction of acid-soluble proteins into the 0.1 *M* citrate–phosphate buffer.

After overnight dialysis of 592 ml of dialysed supernatant against 6 l of saturated ammonium sulphate in 20 mM sodium dihydrogen orthophosphate–sodium hydroxide (pH 6.5), precipitation of enzyme was complete. The precipitated protein was centrifuged as described above. The precipitate was stirred with 60 ml of 0.1 *M* sodium citrate–sodium phosphate buffer (pH 4.4) for 30 min at 4° which disolved the precipitated protein. Removal of residual ammonium sulphate by overnight dialysis against 1 l of 0.1 *M* sodium citrate–sodium phosphate buffer (pH 4.4) resulted in re-precipitation of 60–65% of protein and recovery of 86% of the enzyme in the supernatant. After centrifugation the supernatant was enriched 8-fold in specific activity with respect to the starting extract.

*Determination of the capacity of hydrophobic supports*

The capacity of an adsorbent used for protein chromatography is operationally defined as the protein to support ratio (milligrams of protein per millilitre of packed support) at which 90% of the applied enzyme activity remains bound to the support after elution of non-adherent protein with the application buffer.

The capacity of phenyl-Sepharose columns, equilibrated with 50 m*M* sodium citrate–sodium phosphate buffer (pH 4.4), was tested by the sequential application of partially purified enzyme solutions and monitoring of the eluates. The results are shown in Table I. As can be seen, as much as 75–80 mg of protein per millilitre of support can be applied to the column with 90% retention of enzyme. This compares with a capacity of 5–10 mg of protein per millilitre of DEAE-Sephadex at pH 6.0.

TABLE I

ASSESSMENT OF THE CAPACITY OF PHENYL-SEPHAROSE FOR HEXOSAMINIDASE
Starting buffer: 0.05 *M* sodium citrate–sodium phosphate (pH 4.4).

| *mg protein applied/ml support* | *Units of enzyme activity/ml support* | | | |
|---|---|---|---|---|
| | *Applied* | *Eluted with buffer* | *Eluted with water* | *Eluted with 0.5% Triton X-100* |
| 14 | 600 | 13 | — | — |
| 28 | 1200 | 41 | 0 | 0 |
| 42 | 1800 | 71 | — | — |
| 56 | 2400 | 143 | 0 | 0 |
| 70 | 3000 | 289 | — | — |
| 84 | 3600 | 372 | 0 | 1080 |
| 112 | 4800 | 864 | — | — |
| 140 | 6000 | 1874 | 1620 | 1140 |

The applied enzyme sample contained both hexosaminidase A and B and analysis of the enzymes eluted with the application buffer, with water and with 0.5% Triton X-100 indicates that the binding of hexosaminidase A to the resin is greater than that of hexosaminidase B. At 140 mg of protein per millilitre of support 31% of the applied enzyme elutes with the application buffer. This fraction is entirely hexosaminidase B. The 19% of the sample eluted with Triton X-100 is 80% hexosaminidase A.

Application of protein samples at or close to the capacity of the column is required for the recovery of enzyme activity. The phenyl-Sepharose column, when loaded at a protein to support ratio of 28 mg/ml retains all but 3% of the applied activity after elution with the application buffer. Surprisingly, no additional enzyme is recovered after elution of the resin with 5 column volumes of water followed by 5 column volumes of 0.5% Triton X-100. At an application ratio of 84 mg of protein per millilitre of packed support, elution of the column with the application buffer followed by water failed to elute enzyme activity, but elution with Triton X-100 resulted in the recovery of 30% of the applied sample.

At a ratio of 140 mg of protein per millilitre of support, conditions under which the column is "overloaded", a total of 77% of the applied enzyme could be eluted. It is evident that capacity alone does not define the conditions of optimal efficiency of hydrophobic chromatography. Pilot columns must also be tested in order to determine the application conditions under which the enzyme is recoverable.

The recoverability of applied enzyme is also affected by the choice of the application buffer. Application of enzyme to the phenyl-Sepharose column in 20 m*M* sodium dihydrogen orthophosphate–sodium hydroxide (pH 7.5)–35% saturated ammonium sulphate, using a protein to support ratio of 140 mg/ml, results in elution of only 33% of the applied activity after successive elutions with the application buffer, water and Triton X-100. In the absence of ammonium sulphate the recovery was 100% but the capacity was 10 mg/ml.

The column procedure chosen for step I-4 represents a compromise between capacity and recovery. Details of the application procedure and stepwise elution of the column are given in the legend to Fig. 1. The recovery of hexosaminidase B was 72% and the increase in specific activity was 9-fold.

The capacity of valine-Sepharose for hexosaminidase was estimated by a procedure similar to that employed for the phenyl-Sepharose column except that the column buffer was 0.9 *M* potassium dihydrogen orthophosphate–potassium hydroxide (pH 7.5). A linear descending buffer gradient was used to elute the enzyme. Under these conditions, the capacity was 36–72 mg of protein per millilitre of support. The use of phosphate as an application buffer proved superior to ammonium sulphate. In 20 m*M* potassium dihydrogen orthophosphate–potassium hydroxide (pH 7.5)–35% saturated ammonium sulphate the capacity of the column was less than 18 mg/ml. Gradient elution of valine-Sepharose was used as step II-4. Details are provided in the legend to Fig. 2. No resolution of the A and B isozymes was achieved.

*Further purification of hexosaminidases*

The pooled hexosaminidase-containing fractions eluted from valine-Sepharose were subjected to further purification using a two-step procedure. Using DEAE-Sephadex chromatography at pH 6.0 (steps I-5a and II-5a), hexosaminidase B (iso-

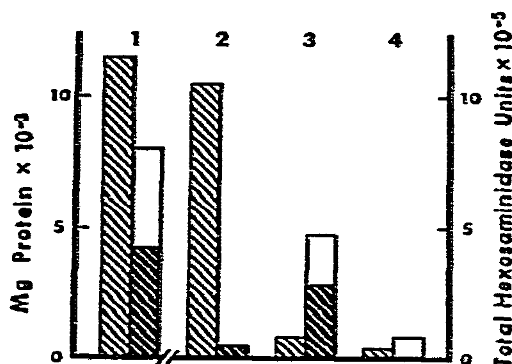


Fig. 1. Stepwise elution of hexosaminidases from phenyl-Sepharose. Phenyl Sepharose was equilibrated with 0.1 *M* sodium citrate–0.2 *M* sodium phosphate buffer (pH 4.4) and packed into a 23 × 3 cm (bed volume 147 ml). The supernatant from step I-3 was diluted with the column buffer to a protein concentration of 5 mg/ml and applied to the column at flow-rate of 60 ml/h. The applied solution is designated (1) on the diagram. The column was eluted with 1500 ml of the same buffer (2), 1500 ml of water (3) and 1500 ml of 0.5% Triton X-100 (4). Lightly hatched bars, total protein; open bars units of total hexosaminidase; heavily hatched portions, hexosaminidase B.

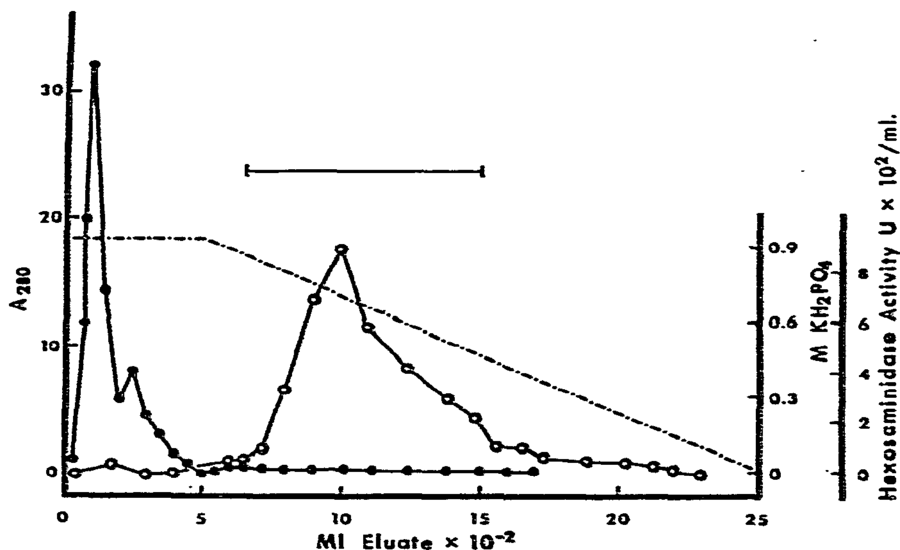


Fig. 2. Reverse gradient elution of hexosaminidases from valine-Sepharose. Valine-Sepharose was equilibrated in 0.9 *M* $KH_2PO_4$–KOH (pH 7.5) and packed into a 21 × 2.7 cm column (bed volume 126 ml). The sample, containing 360,000 units of total hexosaminidase and 6400 mg of protein in a volume of 80 ml, was applied at a flow-rate of 10 ml/h. After elution of protein was virtually complete, a gradient was applied using a 1-l mixing chamber, 0.9 *M* $KH_2PO_4$–KOH (pH 7.5) and a reservoir of deionized water. Fractions of 20 ml were monitored for protein (●) and for hexosaminidase (○). Bar indicates fractions which were pooled.

electric point 6.8–7.3) showed no anionic characteristics and did not bind to the support. The pooled eluate, containing hexosaminidase B but no hexosaminidase A, was adjusted to pH 7.5 and re-chromatographed on DEAE-Sephadex at the higher pH. The enrichment of specific activity was 22-fold. The hexosaminidase B obtained

from step I-5b was lyophilized and resuspended in 20 m*M* sodium dihydrogen orthophosphate–sodium hydroxide (pH 7.0). After re-solubilization only 13% of the protein and 91% of the enzyme activity were recovered. A summary of the two purification procedures is given in Table II.

TABLE II

PURIFICATION PROCEDURES FOR HUMAN LIVER HEXOSAMINIDASE B

| *Procedure* | *Step* | *Material/operation* | *Total activity units × $10^{-2}$* | *Protein (mg)* | *Specific activity (units/mg)* |
|---|---|---|---|---|---|
| I | 1 | Liver supernatant | 4200 | 60.000 | 7 |
| | 2 | Dialysis (pH 4.4) | 4200 | 18.900 | 22 |
| | 3 | Incubation at 37° and pH 4.4 | 4170 | 11.800 | 35 |
| | 4 | Phenyl-Sepharose chromatography | 2915 | 900 | 323 |
| | 5a | DEAE-Sephadex (pH 6.0) | 2720 | 731 | 372 |
| | 5b | DEAE-Sephadex (pH 7.5) | 2204 | 48 | 4800 |
| | 6 | Lyophilization | 2006 | 6 | 33400 |
| II | 1 | Liver supernatant | 4200 | 60.000 | 7 |
| | 2 | Dialysis (pH 4.4) | 4200 | 18.900 | 22 |
| | 3 | $(NH_4)_2SO_4$ precipitation | 3612 | 6.450 | 56 |
| | 4 | Valine-Sepharose chromatography | 2562 | 645 | 397 |
| | 5a | DEAE-Sephadex (pH 6.0) | 1710 | 359 | 476 |
| | 5b | DEAE-Sephadex (pH 7.5) | 1010 | 21 | 4840 |

*Characteristics of purified hexosaminidase B*

The purified hexosaminidase B obtained from steps II-5 and I-6 was subjected to electrophoresis and stained for both protein and enzyme activity. The enzyme prepared using procedure II had a single major protein band with a contaminant estimated to account for about 10% of the protein in the sample. The major band corresponded to the single band obtained after staining half of the gel

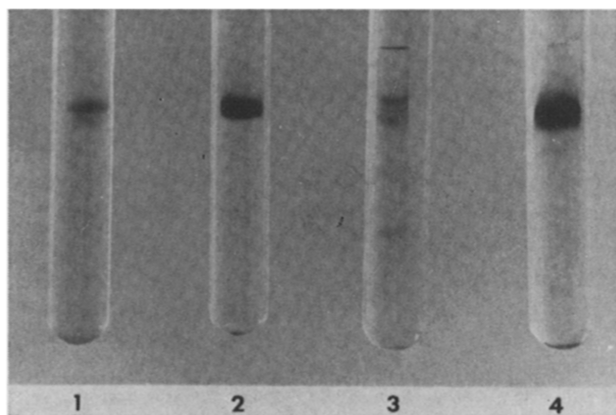


Fig. 3. Polyacrylamide gels: 1, hexosaminidase B purified using procedure I, stained with amido black; 2, hexosaminidase B purified by procedure I, stained with activity stain; 3, hexosaminidase B purified by procedure II, stained with amido black; 4, hexosaminidase B purified by procedure II, stained with activity stain.

with an enzyme activity stain. The enzyme prepared using procedure I shows three protein bands in the region of the gel which corresponds to the single band observed after staining the half-gel with the activity stain. A fourth protein band, representing a contaminant with greater mobility, is also present (Fig. 3).

Hexosaminidase B isolated by either procedure was completely stable to heating for 30 min at 54°, whereas hexosaminidase activity in the starting extract lost 55% of its activity after heating.

## DISCUSSION

The purification procedures reported here demonstrate the utility of hydrophobic chromatography in enzyme purification. Relevant observations on the use of this procedure include the following. (a) The resins have a very high capacity for hexosaminidases[9] and possibly for other lysosomal hydrolases also. (b) Unlike ion-exchange chromatography, the recoverability of enzyme activity in subsequent eluates increases when the column is loaded close to capacity. We speculate that not all hydrophobic sites are equivalent. Some sites, perhaps formed by the proximity of two or more ligands, bind molecules more firmly than others. Molecules bound at such sites cannot be displaced by elution buffers but can be displaced to sites of lower affinity by protein molecules of greater hydrophobicity. The concentration of these molecules increases in proportion to thes ize of the sample. It is only when enzyme is displaced toward sites of lower hydrophobicity that it can be eluted by detergents or descending salt gradients. (c) The phenyl-Sepharose column appears to bind hexosaminidase A more firmly than hexosaminidase B. The use of a gradient of Triton X-100 may possibly effect a complete resolution of the two isozymes.

The combination of solubility steps and ion-exchange chromatography was used together with hydrophobic chromatography to achieve a concentration of 4700-fold. The purified enzyme has properties similar to those of other preparations of human hexosaminidase B[10–14].

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