Separation and Properties of Human Brain Hexosaminidase C

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Hexosaminidase C was separated from human brain supernatant by immunoabsorption of the A and B forms on to a column of immobilized antibody followed by preparative starch-block electrophoresis. There were some differences in the properties of hexosaminidase C preparations after each of these stages, shown by comparison of their heat-inactivation characteristics and filtration through Bio-Gel P-200. The C form prepared by both separation steps had properties which differed markedly from those of the A and B isoenzymes; its molecular weight was much larger, greater than 200 000, it had optimum activity between pH 6 and 7 and could not be successfully eluted from DEAE-cellulose, even with high salt concentrations, or from Sephadex G-200. These results seem to support the proposal that the C form is under a separate genetic control from the others.

Human hexosaminidase (2-acetamido-2-deoxy- β -D-glucoside acetamidodeoxyglucohydrolase; EC 3.2.1.30), a lysosomal hydrolase, was shown by Robinson & Stirling (1968) to exist in two major forms named A and B, which differed in electrophoretic mobility and thermostability, but had similar molecular weights and identical optimum activities at pH 4.5. The properties of the isoenzymes have been further characterized (Okada & O'Brien, 1969; Suzuki & Suzuki, 1970; O'Brien *et al.*, 1971a; Sandhoff & Wässle, 1971). Absence of one or both isoenzymes is associated with a lethal accumulation of GM₂-ganglioside in the brain as in Tay-Sachs disease when hexosaminidase A is absent (O'Brien *et al.*, 1971b) or in Sandhoff's disease when both isoenzymes are absent (Sandhoff *et al.*, 1971).

Hooghwinkel *et al.* (1972) found another form of hexosaminidase, hexosaminidase C, when they examined human brain homogenates by electrophoresis on cellulose acetate paper. This new form appeared to have little or no *N*-acetylgalactosaminidase activity and to be absent from human liver tissue. Poenaru & Dreyfus (1973) confirmed the existence of hexosaminidase C in human brain by electrophoresis on Cellogel and also observed it in liver tissue from human adults and, in higher proportions relative to forms A or B, in embryonic tissue. Hexosaminidase C is not precipitated by antisera to hexosaminidase A and B, so that it may be distinguishable from the other isoenzymes by its immunological as well as by its electrophoretic behaviour (Poenaru *et al.*, 1973). B. Overdijk (unpublished work) has observed that hexosaminidase C has higher activity at pH 7.0 than at pH 4.5.

Hitherto, most of the reported investigations into hexosaminidase C depended on analytical separation of the isoenzymes by electrophoresis followed by treatment of the zymogram with substrate under the appropriate conditions. The present study was carried out in an attempt to prepare hexosaminidase C separated from the other isoenzymes and to investigate its properties.

Experimental

Materials

The following materials were purchased from the suppliers indicated: 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucosaminide (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.); Sephadex G-200 and Sepharose 4B [Pharmacia (G.B.) Ltd., London W.5, U.K.]; Bio-Gel P-200 (V. A. Howe and Co., London S.W.6, U.K.); DEAE-cellulose (DE-23; Whatman Chemical Co., Maidstone, Kent, U.K.); CNBr (Eastman-Kodak Ltd., Kirkby, Lancs., U.K.); ethanolamine and maize starch (BDH Chemicals Ltd., Poole, Dorset, U.K.); Cellogel papers (Reeve-Angel Ltd., London E.C.4, U.K.).

Post-mortem human liver and brain samples, obtained at autopsy, were stored at -20°C . Samples of brain were gifts from Dr. G. J. H. Hooghwinkel, Rijksuniversiteit Leiden, Holland, and Dr. D. Bowen, Institute of Neurology, London, U.K.

Methods

Enzyme assay. Hexosaminidase activity was determined by a modification of the fluorimetric procedure of Robinson *et al.* (1972) as follows: the

enzyme preparation (20 μ l) was incubated with 100 μ l of 0.35 mM - 4 - methylumbelliferyl - 2 - acetamido - 2 - deoxy- β -D-glucosaminide, which had been dissolved in sodium phosphate-citric acid buffer (McIlvaine, 1921), at either pH 4.5 or 7.0. After 30 min at 37°C the reaction was stopped by the addition of 2.0 ml of 0.05 M-glycine previously adjusted to pH 10.4 with 0.2 M-NaOH.

Determination of pH optima. Activity was measured over the pH range 3.5–7.0 in sodium phosphate-citric acid buffer. Enzymes (20 μ l) and buffer (80 μ l) were mixed with aqueous substrate (20 μ l) to give a final concentration of 0.3 mM-4-methylumbelliferyl *N*-acetyl- β -glucosaminide for incubation at 37°C and subsequent fluorescence measurements.

Heat-inactivation. An enzyme preparation in 0.01 M-sodium phosphate buffer, pH 7.0 (20 μ l), was incubated for various times up to 45 min at 55°C. At the end of each incubation time, the tubes were placed in ice until enzyme activity could be assayed at pH 4.5 and 7.0 as described above.

Preparation of antigen. A partially purified preparation containing hexosaminidases A and B was prepared from human liver. An $(\text{NH}_4)_2\text{SO}_4$ precipitate was obtained by the first three steps of the method described by Carroll & Robinson (1973). This precipitate was then dissolved in 0.01 M-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl, to give 50 ml of solution containing 3.4 g of protein. Ethanol was added dropwise to the solution at 10°C and the bulk of the enzyme activity was precipitated between 50 and 60% (v/v) ethanol. This precipitate was redissolved in the 0.01 M-sodium phosphate buffer. This method gave a 40% recovery of the initial activity with a 40-fold increase in specific activity.

Preparation of antibodies. Antisera to the above enzyme preparations were raised in rabbits as described by Carroll & Robinson (1973). Typically, 1.0 ml of antiserum could precipitate from solution enzyme activity equivalent to the hydrolysis of 50 nmol of the test substrate/min.

A crude γ -globulin preparation was obtained by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 50 ml of antiserum (4.1 g of protein) at 4°C to a final concentration of 1.8 M. The precipitate was collected after 30 min centrifugation (3×10^5 g-min), dissolved in 25 ml of 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl ('phosphate-buffered saline'), and reprecipitated in 1.5 M- $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in 0.1 M-sodium citrate buffer (pH 6.5) and then dialysed against the same buffer for 16 h at 4°C, after which time the protein concentration was 60 mg/ml.

Preparation and use of immobilized antibodies. The dialysed globulin fraction was coupled overnight at 4°C with 16 ml of Sepharose 4B activated with CNBr (Steers *et al.*, 1971). Free reactive groups were blocked

by incubation of the gel with excess of 1 M-ethanolamine (pH 8.0) for over 4 h at room temperature (18°C). The gel was washed with 100 ml of 6 M-urea in 0.01 M-sodium phosphate buffer, pH 7.0, followed by exhaustive washing with distilled water for 3 h.

The immobilized antibody thus prepared was used to selectively adsorb hexosaminidases A and B as follows. A 50% (w/v) homogenate of human brain was prepared in phosphate-buffered saline and centrifuged at 10000 *g* for 30 min. Up to 10 ml of the supernatant was then applied to a column (10 cm $\times$ 1 cm diam.) of the immunoabsorption gel, packed and eluted at 4°C with the phosphate-buffered saline. Fractions were collected at a flow rate of 9 ml/h and tested for *N*-acetyl- β -glucosaminidase activity at pH 7.0. Those fractions with greatest activities were pooled and concentrated overnight by vacuum dialysis against 0.04 M-potassium phosphate buffer, pH 6.6. Hexosaminidase species that had bound to the column could be eluted at room temperature with 0.2 M-glycine that had been adjusted to pH 2.8 with conc. HCl. The column was then regenerated for further use by washing with phosphate-buffered saline.

Ion-exchange chromatography. DEAE-cellulose (DE-23) was equilibrated in 0.01 M-sodium phosphate buffer, pH 7.0, and packed into a Pharmacia K9 column (10 cm $\times$ 1 cm diam.). Supernatant (5 ml) from tissue homogenized in 0.01 M-sodium phosphate buffer, pH 7.0, and centrifuged as above was applied and non-adsorbed protein was eluted with the same buffer. Bound proteins were eluted by introducing a gradient of NaCl into the buffer up to a final concentration of 0.4 M.

Gel filtration. Sephadex G-200 or Bio-Gel P-200 were each equilibrated in 0.01 M-sodium phosphate buffer, pH 7.0, packed in Pharmacia K-26 columns (25 cm $\times$ 3 cm diam.) and eluted with this buffer at 9 ml/h.

Electrophoresis. Cellogel electrophoresis was carried out as described by Poenaru & Dreyfus (1973) by using potassium phosphate buffer, pH 6.6 (0.04 M with respect to phosphate), and running strips for 1.5 h at 4°C and 200 V (potential gradient 13.3 V/cm). Starch blocks (20 cm $\times$ 20 cm $\times$ 0.5 cm thick) were prepared by suspending 180 g of maize starch in 180 ml of 0.04 M-potassium phosphate buffer, pH 6.6, as outlined by Kunkel (1954). Protein samples (0.5–1.0 ml) were pipetted into a trough (1 mm wide and 10–16 cm long) cut 5 cm from the cathode end of the block. After electrophoresis overnight at 4°C the block was cut into 0.75 cm-wide strips. The starch was transferred to a sintered-glass filter funnel and the protein content was eluted with the potassium phosphate buffer. The eluted fractions were assayed for hexosaminidase activity and those with greater activity at pH 7.0 than at pH 4.5 were pooled and concentrated.

Protein concentrations were determined by the

method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Separation of human hexosaminidases

When supernatants from homogenates of normal adult brain samples, which had previously been shown to contain hexosaminidase C by electrophoresis, were applied to DEAE-cellulose columns and eluted with 0.01M-sodium phosphate buffer, pH 7.0, two peaks of hexosaminidase were detected in the eluate. The first was in the void volume and was shown, after concentration by vacuum dialysis, to have the electrophoretic mobility of hexosaminidase B. The second peak was eluted at 0.2M-NaCl in the salt gradient and, when concentrated, migrated as a single zone on electrophoresis with the mobility of the A form. No enzyme with the properties of hexosaminidase C could be detected in the eluate even when the salt concentration was increased to 1.0M. It was concluded that this method should be avoided in attempts to isolate hexosaminidase C. Its behaviour on DEAE-cellulose distinguishes the C form from all other reported forms of human hexosaminidase, each of which has been eluted from DEAE-cellulose in good yield.

The separation procedure adopted made use of an immunoadsorption column (step 1) followed by starch-block electrophoresis (step 2). When a portion (10ml) of the supernatant from a normal adult brain homogenate was passed through the column containing bound antibodies to human liver hexosaminidase A and B, the eluate displayed two to three times more activity when assayed at pH 7.0 than at pH 4.5. After concentration of the eluate a single zone of activity, with the mobility of hexosaminidase C, was seen on Cellogel electrophoresis and the preparation was apparently uncontaminated even by traces of A and B forms (Plate 1). When further 10ml portions were passed through the column the ratio of activity assayed at pH 4.5 compared with that measured at pH 7.0 increased in the eluate and Cellogel electrophoresis showed that, although it was relatively enriched in hexosaminidase C, further bands of activity were also present. At first a single new zone with the mobility of hexosaminidase A was detected (Plate 1), but later a zone corresponding to the B form also appeared.

The immunoadsorbent gel was regenerated at room temperature (18°C) by suspending it in 0.2M-glycine buffer, pH 2.8, repacking it and washing with several bed-volumes of the same buffer. When supernatants from brain homogenates were passed through the gel treated in this way the first recoveries of hexosaminidase activity in the effluent again migrated entirely as the C form on electrophoresis. About 40% of the hexosaminidase activity, assayed at pH 7.0, appeared

Fig. 1. Starch-block electrophoresis of first effluent from immunoadsorption column

Starch blocks were prepared in 0.04M-potassium phosphate buffer, pH 6.6, and electrophoresis was carried out overnight at 4°C and 100V. Hexosaminidase activity was measured at pH 4.5 (○) and 7.0 (●) and protein concentrations (□) were determined by the method of Lowry *et al.* (1951).

in the effluent as against less than 2.5% of the activity measured at pH 4.5. The protein content of the column effluent was not significantly different from that of the original homogenate.

For starch-block electrophoresis (step 2) the total effluent from step 1 (10–15ml) was pooled and concentrated to less than 1.0ml by vacuum dialysis against 0.04M-potassium phosphate buffer, pH 6.4. A typical distribution of hexosaminidase activity measured at pH 4.5 and 7.0 after electrophoresis overnight is shown in Fig. 1. Between 80 and 100% of the activity was recovered in a single well-defined zone which also accounted for about 25% of the applied protein. The hexosaminidase C activity of this zone was found to retain its characteristic mobility on Cellogel (Plate 1). When the hexosaminidase C from step 1 was obtained contaminated with the more slowly migrating forms, it could be separated from them by the starch-block electrophoresis. Three distinct zones of hexosaminidase activity were found in these starch-block-electrophoresis preparations, the fastest of which was shown to consist only of the C form by Cellogel electrophoresis.

Heat-inactivation

Hexosaminidase A has been distinguished from the B form by its more ready denaturation on gentle heating. The effects of incubation at 55°C in sodium phosphate buffer, pH 7.0, on various human hexosaminidase preparations were compared (Fig. 2).

Liver hexosaminidase B, separated from other forms by DEAE-chromatography, showed no loss of activity in 30min. Liver hexosaminidase A and brain hexosaminidase C activities from step 2 decayed exponentially at closely similar rates. By contrast the hexosaminidase C preparation from step 1 showed more complex non-linear inactivation characteristics even though it appeared identical with the step 2 preparations on Cellogel electrophoresis.

Gel filtration of human brain extracts

Supernatants from human brain homogenates were filtered through Bio-Gel P-200 and the eluates assayed at pH4.5 and 7.0 (Fig. 3). Two peaks of activity measurable at pH7.0 were found and these were pooled separately and concentrated. On Cellogel electrophoresis the activity of the first peak migrated as a single zone with the mobility of hexosaminidase C, and the second peak consisted mainly of hexosaminidase A and B with slight amounts of the C form. Most of the activity that could be assayed at pH4.5 was eluted with the second peak with a shoulder which corresponded in position to the first peak. By integration of the areas under

Fig. 2. Heat-inactivation of human hexosaminidases

Enzyme preparations were preincubated for various times at 56°C in 0.01M-sodium phosphate buffer, pH7.0. Activities were then measured in phosphate-citric acid buffer at either pH4.5 or 7.0, with liver hexosaminidase B measured at pH4.5 (▼), liver hexosaminidase A at pH4.5 (■), brain hexosaminidase C from step 2 at pH7.0 (●), and brain hexosaminidase C from step 1 at pH7.0 (○).

Fig. 3. Gel filtration of human brain extract on Bio-Gel P-200

Columns (25cm×3cm diam.) of Bio-Gel P-200 were eluted at 9ml/h with 0.01M-sodium phosphate buffer, pH7.0, and hexosaminidase activity was measured at pH7.0 (●) and 4.5 (○). V_e , Elution volume; V_0 , void volume.

these peaks hexosaminidase C activity, assayed at pH7.0, was calculated to be equal to approx. 8% of the combined hexosaminidase A and B activities, assayed at pH4.5.

It is apparent from Fig. 3 that hexosaminidase C has a higher molecular weight than the other forms of human hexosaminidase and in order to estimate this more precisely the separated hexosaminidase C preparation from step 2 was applied to a calibrated column (Andrews, 1965). A single symmetrical peak was eluted in the exclusion volume of the column indicating that its apparent molecular weight was greater than 200000 (Fig. 4a). For comparison the partially purified liver hexosaminidase B preparation was also applied to the column and eluted in a volume which corresponded to a molecular weight of between 120000 and 140000. The peak fractions of hexosaminidase C were concentrated and electrophoretic examination showed that its mobility was unaltered by filtration through Bio-Gel P-200 (Plate 1).

The behaviour on Bio-Gel P-200 of the hexosaminidase C preparation from step 1 (Figs. 4a and 4b) was in marked contrast with that of the preparation from step 2 just described. Although the main peak of activity was still eluted in the void volume, there was a marked trail of activity spread over a volume corresponding to the molecular-weight range of 160000

EXPLANATION OF PLATE I

Cellogel electrophoresis of human brain hexosaminidases

Cellogel strips were run in 0.04 M-potassium phosphate buffer, pH 6.6, for 1.5 h at 2000 V. Enzyme activities were detected by incubation with 4-methylumbelliferone substrate followed by 0.05 M-glycine buffer, pH 10.4. (a) Supernatant from human brain (i); first effluent from immunoadsorption chromatography, step 1 (ii); hexosaminidase C zone from starch-block electrophoresis, step 2 (iii); extract from human spleen (iv). (b) Supernatant from human brain (i); later effluent from immunoadsorption column (ii); hexosaminidase C from starch-block electrophoresis (iii); peak (iv) and trail (v) pooled fractions from Bio-Gel P-200 filtration of hexosaminidase C from step 1; hexosaminidase C from step 2 after filtration through Bio-Gel P-200 (vi); human spleen extract (vii).

Fig. 4. Gel filtration of isolated human hexosaminidases on Bio-Gel P-200

Conditions for the column were identical with those described for Fig. 3. (a) Liver hexosaminidase was prepared as described in the text and its activity in the eluate measured at pH 4.5 (○). Hexosaminidase C was prepared by immunoabsorption chromatography and starch-block electrophoresis and its activity was measured at pH 7.0 (●). (b) Eluate from the step 1 preparation of hexosaminidase C was assayed at pH 4.5 (□) and 7.0 (■). Eluate from the step 2 preparation was also assayed at pH 4.5 (○) and 7.0 (●).

Fig. 5. pH-activity profiles of human hexosaminidases

Activities were measured in a range of phosphate-citric acid buffers between pH 3.5 and 7.0, with a final substrate concentration of 0.35 mM. (a) Liver hexosaminidase B (■); brain hexosaminidase C from step 1 (○); brain hexosaminidase C from step 2 (●). (b) Effect of variation of pH on activity of hexosaminidase from step 1 (●) and from step 2 (○) were compared after filtration on Bio-Gel P-200.

down to 70000. In this trial, hexosaminidase activity appeared greater when assayed at pH 4.5 than at pH 7.0 and overall there was a threefold increase in the total activity at pH 4.5 in the eluate when compared with that of the sample applied to the column. The peak and trail fractions were pooled and concentrated separately. Cellogel electrophoresis showed that only hexosaminidase C was present in the peak fractions, whereas in the trail fractions additional ones were observed with mobilities similar to those of hexosaminidase A and B (Plate 1).

The recovery of hexosaminidase C activity from step 2 after filtration through Bio-Gel P-200 was

always very high (90–100%). However, when a similar preparation was applied to a Sephadex G-200 column of the same dimensions and eluted under identical conditions no hexosaminidase activity could be detected in the eluate when measured at pH 7.0 or 4.5.

pH-activity profiles

The activities of several human hexosaminidase preparations were compared in sodium phosphate-citric acid buffers in the pH range 3.5–7.0 (Fig. 5a). Liver hexosaminidase B gave a symmetrical pH-activity curve with optimum activity at pH 4.5. The

supernatant of a human brain homogenate gave a very similar curve except that activities were relatively higher on the alkaline side of the optimum. However, the shape of the curve would not of itself have suggested the presence of a hexosaminidase component with optimum activity about pH 7.0. The hexosaminidase C preparations obtained after either step 1 or step 2 both gave essentially the same pH-activity profile with maximum activity between pH 6.0 and 7.0.

The elution pattern obtained when the hexosaminidase C activity from step 1 was filtered through Bio-Gel P-200 (Fig. 4b) suggested the presence of a component with optimum activity below pH 7.0. To test this possibility the pH-activity profiles of hexosaminidase C preparations obtained from step 1 and from step 2 were redetermined after each had undergone gel filtration through Bio-Gel P-200 (Fig. 5b). The step 2 preparation still showed the characteristic hexosaminidase C profile with maximum activity between pH 6.0 and 7.0, but the step 1 preparation gave a complex pH-activity profile with a sharp optimum at about pH 5.25 and a plateau between pH 6.0 and 7.0. Such a profile suggests the presence of more than one component in this hexosaminidase C preparation although it migrated as a single fast-moving band on Cellophane electrophoresis (Plate 1).

Discussion

This study shows that hexosaminidase C may be prepared free from the A and B isoenzymes by immunoadsorption chromatography, followed if necessary by starch-block electrophoresis. The properties of such preparations differ markedly from those of hexosaminidases A and B and those of the other forms of hexosaminidase recently characterized, namely serum hexosaminidase P (Stirling, 1972) and the intermediate forms I_1 and I_2 (Price & Dance, 1972). Hexosaminidase C has a molecular weight greater than 200 000, whereas these other forms have molecular weights in the range 120 000–140 000. Optimum activity for form C lies between pH 6 and 7, although it is about pH 4.5 for the other isoenzymes. At the lower pH, form C contributes little to the total brain hexosaminidase activity and its presence may therefore have been overlooked in previous investigations. Hexosaminidases A, B, P and the intermediate forms are all readily prepared by ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-200, but form C could not be separated by either of these techniques. The high net charge of the isoenzyme may account for the lack of success in eluting it from DEAE-cellulose, since it may be firmly bound or denatured under these conditions.

In the course of our purification it was found that

the hexosaminidase activities eluted from the immunoadsorption column, although appearing homogeneous on electrophoresis, showed signs of heterogeneity on examination by other criteria. Decay of activity on heating was not exponential and gel filtration revealed components with apparently lower molecular weights than hexosaminidase C and electrophoretic mobilities similar to those of the A and B forms, although before filtration these forms were not evident on electrophoresis. It may be that the preparations obtained from the immunoadsorption column contain a minor and variable proportion of hexosaminidases bound to acid mucopolysaccharides. It has been shown with other glycosidases that artifacts with rapid electrophoretic mobilities can be produced in this way (Kint, 1973). If this occurs with brain hexosaminidases A and B, then the binding clearly alters their serological properties since they are not bound by the immunoadsorption column. The association between the enzyme and the acid mucopolysaccharides would then appear to be disrupted during the process of starch-block electrophoresis. It would be necessary to assume that the binding also drastically decreases hexosaminidase activity at pH 4.5, since the pH-activity profiles of the preparations from step 1 and step 2 are similar (Fig. 5a).

Human hexosaminidase can now be seen to be more closely comparable with other glycosidases which have some forms that are most active at acid pH values and are usually found predominantly in the lysosomal fraction of a tissue, and other forms more active at neutral pH values and mainly in the microsomal fraction. This pattern is shown by β -galactosidase in rat kidney (Furth & Robinson, 1965) and human liver (Ho & O'Brien, 1971a), by β -glucosidase in human spleen (Ho & O'Brien, 1971b) and by α -glucosidases in various human tissues (Hers, 1963). The similarity between human hexosaminidases and the α -mannosidases of human liver (Carroll *et al.*, 1972) and bovine tissues (Phillips *et al.*, 1974) is especially close in that the forms with neutral pH optima have higher molecular weights than the acid forms in each case. By analogy with the rabbit kidney α -mannosidase (Marsh & Gourlay, 1971) and other glycosidases, it may be suggested that the human hexosaminidases with acid pH optima are lysosomal hydrolases, whereas hexosaminidase C is located in the microsomal fraction and performs an independent function. The frequent persistence of the microsomal type of activities in cases of lysosomal enzyme deficiency, taken with the functional, size and serological differences between the microsomal and the lysosomal activities, suggest that they are under separate genetic control.

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