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The Separation of Bovine Brain β-*N*-Acetyl-D-hexosaminidases

ABNORMAL GEL-FILTRATION BEHAVIOUR OF β-*N*-ACETYL-D-GLUCOSAMINIDASE C

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Bovine brain tissue was extracted and the 50000*g* supernatant was separated by electrophoresis, DEAE-Sephadex chromatography and gel filtration on Sephadex G-200 and Bio-Gel P-200. The electrophoretic separation showed that the β-*N*-acetyl-D-hexosaminidases (hexosaminidases) of bovine brain tissue were composed of four different fractions. Two fractions (A and B) exerted both glucosaminidase and galactosaminidase activity, a third fraction (C) showed only glucosaminidase activity, whereas a fourth form (D) with specificity towards the galactosaminide moiety was found to be present. DEAE-Sephadex chromatography at pH7.0 showed that the B form was eluted with the void volume, whereas the A and D forms could be eluted in one peak by raising the salt concentration. The C form could not be detected in the eluate. Gel filtration on Sephadex G-200 showed that the B, A and D forms had almost equal molecular weights. In this case also the C form could not be detected in the column eluates. Gel filtration on Bio-Gel P-200 revealed that the C form was eluted with the void volume.

Robinson & Stirling (1966, 1968) described the separation of human spleen hexosaminidases into two multiple forms, A and B, by means of starch-gel electrophoresis and DEAE-cellulose column chromatography, The B form migrated to the cathode on starch gel at pH6.0, whereas the A form migrated to the anode. With DEAE-cellulose chromatography the enzyme could be separated into two forms, a 'free' one which was eluted with the void volume and which appeared to be the B form on electrophoresis, and a 'bound' one which could be set free from the ion-exchanger by increasing the salt concentration of the elution buffer. Electrophoresis of this form showed that it had the mobility of the A form. Frohwein & Gatt (1967*a*) separated the bovine brain enzyme into three fractions by using a centrifugation technique. One fraction had only glucosaminidase activity, a second fraction exerted only galactosaminidase activity, whereas a third fraction had both activities and was therefore named hexosaminidase. Robinson *et al.* (1972) found three fractions on starch-gel electrophoresis in bovine brain. DEAE-cellulose chromatography also showed a third minor peak, but all peaks showed both glucosaminidase and galactosaminidase activity.

Isoelectric focusing resulted in a greater heterogeneity (Sandhoff, 1968; Robinson *et al.*, 1972). Hooghwinkel *et al.* (1972) reported the presence of a third form (hexosaminidase C) in human and in bovine brain on cellulose acetate paper electrophoresis, which was also found by Poenaru & Dreyfus (1973) in other human tissues. The form C appeared to have a specific glucosaminidase activity. On Sephadex G-200 gel filtration the A and B forms of the bovine brain enzyme were eluted together, corresponding to a molecular weight of 150000 (Robinson *et al.*, 1972), but the C form was not found in the column eluates (Braidman *et al.*, 1974*a*). Ion-exchange chromatography on DEAE-Sephadex (Overdijk, 1975) or DEAE-cellulose (Braidman *et al.*, 1974*a*) also resulted in the disappearance of this fraction C, both in human and in bovine brain extracts.

The present paper describes the electrophoretic separation of the bovine brain enzyme and its separation behaviour on Sephadex G-200 and Bio-Gel P-200 columns, together with a peculiar result for the same separation on an 'aged' Sephadex G-200 column.

Experimental

Materials

The following materials were purchased from the suppliers indicated: 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside and 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-galactopyranoside (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.); Sephadex G-200, DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden); Bio-Gel P-200 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.); Sepraphore III cellulose acetate

paper (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.); Cellogel (Chemetron, Milan, Italy); special agar noble (Difco Laboratories, Detroit, Mich., U.S.A.). All other reagents were of analytical grade.

Bovine brain tissues were obtained from the Leiden slaughterhouse immediately after the animals were killed.

Methods

Enzyme assay. The activity of glucosaminidase and galactosaminidase was determined as follows. The substrate solution was prepared by adding 5 parts of 0.05M-Tris–0.1M-KCl adjusted to pH 7.0 with concentrated HCl (Tris–KCl) to 2 parts of a Na_2HPO_4–citric acid buffer of a pH value as indicated in the text (McIlvaine, 1921); in this mixture the solid substrates were dissolved to a final concentration of 1.2mM. The incubation mixture was composed of 0.1ml of enzyme solution and 0.5ml of substrate solution. The incubations were carried out at 37°C for 30min for the glucosaminidase activity and for 120min for the galactosaminidase activity. The reaction was stopped with 2ml of a buffer containing 0.25M-glycine adjusted to pH 10.5 with 0.25M-Na_2CO_3 and the liberated 4-methylumbelliferone was measured with a Vitatron MPS photofluorimeter with a primary filter U10 (maximum transmission at 365nm) and a secondary filter U8 (maximum transmission at 410nm).

Preparation of tissue extracts. The brain grey matter was separated from white matter and homogenized with a Potter–Elvehjem homogenizer to a 50% (w/v) homogenate in 0.05M-Tris–0.1M-KCl buffer, pH 7.0. After centrifugation at 50000*g* for 1h in a Sorvall RC 2-B refrigerated centrifuge (SS 34 rotor; 20000rev./min) the supernatant was used as the enzyme solution, diluting where necessary with the Tris–KCl used for homogenization.

Preparation of the chromatography columns. The column materials were swollen according to the instructions of the suppliers. The Sephadex G-200 and Bio-Gel P-200 were swollen in 0.05M-Tris–0.1M-KCl, pH 7.0, whereas the DEAE-Sephadex was swollen in 0.05M-Tris–0.05M-KCl, pH 7.0. Before filling the columns the gel suspension was decanted five times to remove the fines and then de-aerated. For Sephadex G-200 and Bio-Gel P-200 the column dimensions were 45cm × 2.5cm diam. and 100cm × 2.5cm respectively. The DEAE-Sephadex columns were 30cm × 1.5cm diam. For gel filtration the flow rate was 8.8ml/h and for DEAE-Sephadex chromatography an operating pressure of 25cm of water was used; fractions (5ml) were collected. The sample volume was less than 2% of the column volume for gel-filtration purposes, and for ion-exchange chromatography 4ml of the 50% (w/v) tissue extract was applied to the column. After 6h of elution with the starting buffer, the KCl concentration was increased linearly up to 0.5M with the aid of a linear gradient-mixer.

Electrophoresis. Sepraphore III electrophoresis in an acetate buffer was done as described earlier (Hooghwinkel *et al.*, 1972). Cellogel electrophoresis was performed by the method of Poenaru & Dreyfus (1973), and agar-gel electrophoresis was done as follows. The agar was mixed with 0.08M-citric acid–0.08M-sodium phosphate buffer, pH 8.0, to a 0.9% (w/v) mixture. This was heated to boiling and then cooled down to 50°C. The warm gel (35ml) was poured on to four glass plates (2.5cm × 7.5cm) resting on a thin layer of agar in a 14cm diam. Petri dish. After 24h the plates were ready for use. The enzyme samples were applied in a small incision and the separation was performed at 120V for 1h. The separation was made visible by incubating the plates with a strip of filter paper, soaked in a 0.1M McIlvaine (1921) buffer solution saturated with substrate, on top of the agar layer. After incubation for 10–20min at room temperature the faint enzyme bands were made clearly visible by holding the plates in NH_3 vapour for about 20s, as was also described for the Sepraphore electrophoresis (Hooghwinkel *et al.*, 1972), and illuminating them with a u.v. lamp (366nm).

Results

Electrophoresis

As described earlier (Hooghwinkel *et al.*, 1972), the separation on Sepraphore III cellulose acetate paper gives three enzyme bands. The fastest-migrating band had both glucosaminidase and galactosaminidase activities, a slower-moving band showed specific galactosaminidase activity and the slowest band had glucosaminidase activity only. We originally denoted them B, A and C respectively (Fig. 1*a*). Agar-gel electrophoresis gives other results (Fig. 1*b*). At pH 8.0 there were two bands with glucosaminidase and galactosaminidase activity moving to the cathode, and also two bands moving to the anode, of which the fastest-moving one was a specific galactosaminidase and the other a specific glucosaminidase. These two anodal-moving bands with a very small migration difference proved to be the A and C bands originally described by Hooghwinkel *et al.* (1972), whereas the two bands moving to the cathode on agar plates both migrated with the same velocity on Sepraphore III paper. These facts were confirmed by re-electrophoresis of paper-separated enzyme bands on agar gel and vice versa.

Another proof of the presence of the four forms of the enzyme was heat-inactivation. On Sepraphore III electrophoresis the C form of the glucosaminidase was absent from a brain extract that had been heated

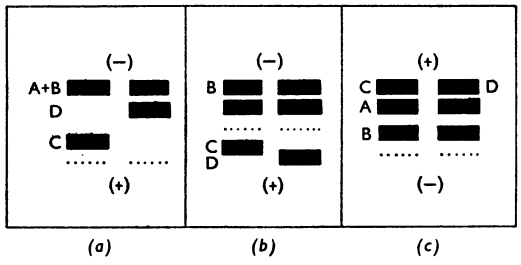

Fig. 1. *Schematic representation of the electrophoretic separation of bovine brain hexosaminidases*

(*a*) Sepraphore III cellulose acetate electrophoresis; (*b*) agar-gel electrophoresis; (*c*) Cellogel cellulose acetate electrophoresis. The left patterns of each pair represent glucosaminidase activities, the right patterns are of the galactosaminidase activities. Experimental details are described in the text.

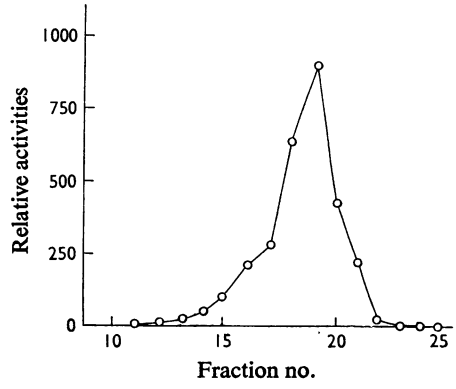

Fig. 2. *Gel filtration on Sephadex G-200 of bovine brain glucosaminidase*

Column dimensions were 45cm×2.5cm. Fraction volume was 6.6ml. Activities were determined at pH6.0. The shape of the curve is independent of the pH of the enzyme assay. The plotted relative activities are the fluorescence values, measured as described under 'Methods'.

for 2h at 50°C. In agreement with this was the disappearance of the slowest anodal-moving fraction on agar gel. On Cellogel electrophoresis in 0.04M-potassium phosphate buffer, pH6.5, three fractions could be seen all moving to the anode when the extract was applied at the cathode (Fig. 1*c*). In the glucosaminidase lane the fastest-moving fraction disappeared when heated extract was used, whereas the corresponding band in the galactosaminidase lane remained active.

Gel filtration

Sephadex G-200 chromatography of a 50% (w/v) extract from bovine brain resulted in a single peak in the elution profile (Fig. 2), regardless of whether the column eluates were assayed for glucosaminidase or galactosaminidase activity. However, electrophoretic examination of the fractions revealed that all multiple forms except glucosaminidase C were present. We have demonstrated (Braidman *et al.*, 1974*a*) the neutral pH optimum of this fraction, but even at pH7.0 no change in the elution profile of the Sephadex G-200 chromatographic separation could be found, nor could it be detected in the electrophoretic pattern of the column fractions at this pH. For some time the disappearance of glucosaminidase C on gel filtration remained a problem. Braidman *et al.* (1974*b*) reported, however, that with the aid of Bio-Gel P-200 the C fraction in human brain tissue extracts could be separated from the other forms.

Bio-Gel P-200 gel filtration of bovine brain extracts gave us the same results (Fig. 3). Glucosaminidase C is present in the void volume of the column as a fraction with mol.wt.$\geqslant$200000. Moreover, Fig. 3 shows that the activity of fraction C at pH6.0 is more pronounced than at lower pH. This agrees with earlier findings on the pH optimum of this fraction (Braidman *et al.*, 1974*a*; Overdijk *et al.*, 1974).

When the tissue extract was passed over an aged Sephadex G-200 column that had been standing for about 10 months without running, the same results were obtained as with the Bio-Gel P-200 filtration (Fig. 3).

The apparent molecular weights of the two peaks, obtained by the method of Andrews (1965), were lower than when a normal Sephadex column was used and were of the same order of magnitude as the results with Bio-Gel P-200 (140000$\pm$10000).

Ion-exchange chromatography

Anion-exchange chromatography with a DEAE-Sephadex A-50 column resulted in two peaks of activity (Fig. 4). The first peak, which was eluted with the void volume, had a mobility on electrophoresis comparable with that of form B of the hexosaminidases, whereas the peak that was displaced from the column with increased salt concentration contained the hexosaminidase A and the galactosaminidase D. The C form could not be detected in any fraction, even when the salt concentration was increased to 1M or higher.

Since a DEAE derivative of the polyacrylamide gels such as Bio-Gel was not available, we were not able to see if the C form could be separated on such a column. As stated in the introduction, DEAE-cellulose chromatographic separation of human brain extract, like DEAE-Sephadex chromatography, did

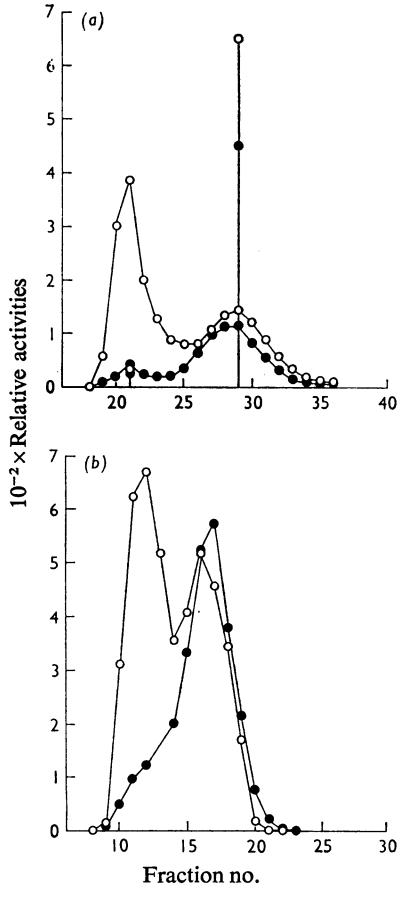

Fig. 3. *Gel filtration of bovine brain glucosaminidase and galactosaminidase (a) on Bio-Gel P-200 and (b) on an 'aged' Sephadex G-200 column*

(*a*) Column dimensions were 100cm×2.5cm. Fraction volume was 6.6ml. ○, Glucosaminidase activity; ●, galactosaminidase activity. For assay details, see under 'Methods'. The curves represent activities at pH6.0. For comparison the activities at pH4.2 of fraction numbers 21 and 29 are given by the vertical bars. (*b*) Column dimensions were 45cm×2.5cm. Fraction volume was 6.6ml. Activities were determined at pH6.0. ○, Glucosaminidase activity; ●, galactosaminidase activity. The plotted relative activities are the fluorescence values, measured as described under 'Methods'.

not separate out the C form either (Braidman *et al.*, 1974*a*).

Discussion

In the present study the separation of the hexosaminidases from bovine brain tissue has been described with special regard to the abnormal gel-filtration behaviour of glucosaminidase C.

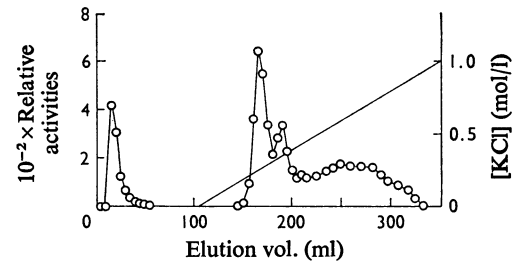

Fig. 4. *Elution pattern of glucosaminidase from DEAE-Sephadex A-50*

Column dimensions were 30cm × 1.5cm. For experimental details see under 'Methods'. The plotted relative activities are the fluorescence values, measured as described under 'Methods'. ○, Relative activity; ——, KCl concn.

It appeared that Sephadex G-200 was not suitable as a separation medium for this form of the enzyme, since the enzyme form could not be detected in the column fractions. Bio-Gel P-200, however, can be used to separate the enzyme form C and to estimate its molecular weight, as was first described by Braidman *et al.* (1974*b*) for the human brain enzyme.

The identity of the C form of glucosaminidase from bovine brain could be established in several ways. First, the neutral character of the enzyme form as shown in Fig. 3 agrees with earlier results for both the human and the bovine brain enzyme (Overdijk *et al.*, 1974; Overdijk, 1975; Braidman *et al.*, 1974*a*). Secondly, the electrophoretic mobility of the enzyme present in these fractions was shown to be identical with that of glucosaminidase C. Thirdly, the relative inactivity of the enzyme towards the synthetic galactosaminide substrate was a proof for the identity of it.

Frohwein & Gatt (1967*a*) and Hooghwinkel *et al.* (1972) described the presence of a glucosaminidase and a galactosaminidase together with an enzyme form with both activities in bovine brain. Frohwein & Gatt (1967*a*) used a centrifugation technique for the separation of these forms, whereas Hooghwinkel *et al.* (1972) separated them by means of Sepraphore III cellulose acetate electrophoresis.

In the present study a combination of three different electrophoresis systems was used, which resulted in a separation into four separate forms, of which two have to be denoted as hexosaminidases because they hydrolyse both synthetic substrates. The DEAE-Sephadex chromatography results indicate that these last two enzymes are, according to the usual nomenclature of the hexosaminidases, the A and B forms. The galactosaminidase might therefore be named D. Gel-filtration studies indicate that this D form has an almost equal molecular weight to the A and B forms, since the activity is present in the same peak as the other two forms (Fig. 3).

An aged Sephadex G-200 column gave the same separation results as a Bio-Gel P-200 column, separating the C form of the enzyme from the other three forms. The only difference between this column and our regular G-200 columns was that the former had been out of use for 10 months and stored at −4°C in the presence of chlorhexidin (0.002%), being given a 2-day flow of the Tris–KCl elution buffer before being put back into use. Protein adsorption by the Sephadex as such cannot be the reason for the loss of glucosaminidase C. The column had been used for so many separations of similar samples that the possible adsorption capacity of the gel would have been exceeded.

A possible explanation for this phenomenon is that the Sephadex material had been changed during storage in such a way that the inactivating properties with respect to glucosaminidase C had been lost, so that it becomes comparable with a Bio-Gel P-200 medium. Although the nature of this change is unclear, these results are important in the separation of acid hydrolases and other glycoproteins on this medium. Poor recoveries or complete disappearances can be expected on behalf of these results also for (glyco)proteins other than glucosaminidase C. This phenomenon may also apply to DEAE-Sephadex and CM-Sephadex and DEAE- or CM-cellulose.

The rôle of the different enzyme forms in the catabolism of the glycosphingolipids is unknown. Frohwein & Gatt (1967*b*) reported that the two synthetic substrate-specific enzyme forms, i.e. the galactosaminidase and the glucosaminidase fractions, were unable to hydrolyse lipid substrates such as trihexosylceramide, globoside and GM_2-ganglioside, whereas their hexosaminidase fraction was able to hydrolyse both lipid and non-lipid substrates. However, Li *et al.* (1973) found that the presence of a heat-stable factor greatly enhanced the hydrolysis of GM_2-ganglioside by hexosaminidase A. Frohwein & Gatt (1967*b*) reported incubation times of 18–24h to obtain a reasonable hydrolysis. It is not known whether the addition of the heat-stable factor of Li *et al.* (1973) activates one or both specific enzyme forms.

Finally, Frohwein & Gatt (1967*b*) suggested that the hexosaminidase fraction could be composed of two enzyme forms because the shape of the pH-optimum curve for the lipid and the non-lipid substrate differed. This may not be surprising in view of the fact that their preparation must have been heterogeneous and contained both hexoasminidases A and B.

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