Studies on Human *N*-Acetyl- β -D-hexosaminidase C Separated from Neonatal Brain

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(Received 22 December 1975)

Human brain hexosaminidase C was separated from isoenzymes A and B by Sephadex G-200 gel filtration. Properties of the enzyme were studied, particularly its isoelectric-focusing profile, pI 4.80. These findings indicate that hexosaminidase C is identical with the major residual component of Sandhoff fibroblasts with respect to substrate specificity, pI and activity pH optimum.

Human *N*-acetyl- β -D-hexosaminidase (EC 3.2.1.30) occurs in two major forms (A and B) that have a common molecular weight, activity pH optimum and K_m . The enzymes differ, however, with respect to their thermal stabilities and electrophoretic mobilities (Robinson & Stirling, 1968), the A form being more heat-labile and electrophoretically more anodic than the B form. Much attention has been paid to these isoenzymes because of their involvement in the GM₂-gangliosidosis. In Tay-Sachs disease (GM₂-gangliosidosis type B) hexosaminidase A activity is deficient (Okada & O'Brien, 1969), and in Sandhoff's disease (GM₂-gangliosidosis type O) hexosaminidases A and B are missing (Sandhoff *et al.*, 1968).

Other forms of hexosaminidase have been reported (Price & Dance, 1972; Stirling, 1972), but recently a neutral isoenzyme, hexosaminidase C, which is electrophoretically more anodic than form A, has also been described (Hooghwinkel *et al.*, 1972; Poenaru & Dreyfus, 1973; Braidman *et al.*, 1974).

It has been claimed (Ropers & Schwantes, 1973; Galjaard *et al.*, 1974; Broadhead & Besley, 1975) that residual activity in Sandhoff's disease can be attributed to hexosaminidase C activity. However, it has also been proposed (Ikonne *et al.*, 1975; Beutler *et al.*, 1975) that this residual activity is due to another isoenzyme, namely hexosaminidase S. The present paper describes properties peculiar to brain hexosaminidase C and shows that with respect to its substrate specificity, activity pH optimum and isoelectric-focusing profile it is identical with the major residual component of Sandhoff fibroblasts (Broadhead & Besley, 1975).

Experimental

Human brain from an anoxic stillborn infant was used within a few hours of death.

The following materials were obtained as indicated: Sephadex G-200 (Pharmacia, London W.5, U.K.); Ampholine (LKB, Stockholm, Sweden); 4-methyl-

umbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside, 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-galactopyranoside (Koch-Light Laboratories, Colnbrook, Bucks., U.K.); Cellogel (Reeve-Angel, London E.C.4, U.K.).

Hexosaminidase activity was determined as follows. Enzyme sample (50 μ l) was incubated for 30 min with substrate (50 μ l) comprising of 4 mM-glucosaminide conjugate, or where indicated 1 mM-galactosaminide conjugate, in sodium phosphate/citric acid buffer, pH 4.5 (at 37°C) (McIlvaine, 1921), containing 0.1% (v/v) Triton X-100 and 0.02% (w/v) NaN₃. Hexosaminidase C activity was determined under similar conditions but at pH 5.5. The reaction was stopped with 0.1 M-Na₂CO₃ (0.3-2 ml), and the released 4-methylumbelliferone was determined fluorimetrically (Butterworth *et al.*, 1972).

Gel filtration was performed on a column (90 cm $\times$ 4.4 cm diam.) of Sephadex G-200, aged 12 months (see Overdijk *et al.*, 1975), and equilibrated at 4°C with 10 mM-sodium phosphate buffer, pH 6.0 (Gomori, 1975), containing 0.1 M-NaCl, 0.1% (v/v) Triton X-100 and 0.02% (w/v) NaN₃. Fresh brain (80 g) was homogenized in eluting buffer (100 ml) in a Silverson homogenizer. After centrifuging at 900g for 30 min (4°C), the supernatant was concentrated tenfold by vacuum dialysis and centrifuged at 10000g for 30 min (4°C), and the supernatant (2 ml) was applied to the column. At an ascending flow rate of 24 ml/h, eluted fractions (8.1 ml) were collected and assayed.

Isoelectric focusing was performed at 4°C in J-tubes (Godson, 1970) of 10 ml working capacity and in a linear sucrose gradient containing 1% (w/v) Ampholine and 0.1% (v/v) Triton X-100. With the cathode uppermost, a voltage of 400 V was applied for 18 h; on completion, fractions (0.3 ml) were collected and the pH (at 4°C) of each was determined immediately.

Electrophoresis was carried out on Cellogel strips by the method of Poenaru & Dreyfus (1973).

Results and Discussion

Hexosaminidase C activity of fresh brain extract was separated from components A and B by Sephadex G-200 gel filtration (Fig. 1). The enzyme was eluted immediately after the void volume, and its activity was demonstrated at pH 5.5 but not at pH 4.5, the activity pH optimum of hexosaminidases A and B in peak II. The two enzyme peaks were separately pooled, concentrated to 5 ml by vacuum dialysis and stored at -70°C for subsequent characterization.

The identity of hexosaminidase C was confirmed by studies on its activity pH optimum, electrophoretic mobility and substrate specificity. In sodium phosphate/citric acid buffer, optimum activity was at pH 5.5–6.0; at pH 4.5 only 11% of the maximum activity could be detected. The C component ran as a single activity band on Cellogel electrophoresis with an anodic mobility greater than that of hexosaminidase A. Electrophoresis of the second Sephadex peak resulted in the two activity bands of hexosaminidases A and B. No activity was seen in the position of hexosaminidase C. However, electrophoresis of the original brain extract had resulted in all three bands of enzyme activity.

Compared with forms A and B, hexosaminidase C exhibited lower galactosaminidase activity, and, under standard assay conditions at pH 5.5, glucosaminidase

activity was 31 times greater than galactosaminidase activity. This specificity was also shown by the low affinity of hexosaminidase C towards the galactosaminide substrate. The apparent K_m values with respect to the 4-methylumbelliferyl conjugates were determined for enzyme peaks I and II at their respective activity pH optima. The apparent K_m values for hexosaminidase C were 0.05 mM and 0.81 mM for glucosaminide and galactosaminide conjugates respectively. These values contrast with those obtained for peak II hexosaminidases A and B, which were 0.88 and 0.116 mM respectively.

The above properties are consistent with those expected for hexosaminidase C activity (Braidman *et al.*, 1974; Penton *et al.*, 1975).

Isoelectric focusing of brain hexosaminidase C, in the range pH 3.5–10, produced (Fig. 2a) a single activity peak, pI 4.77. When assayed at pH 4.5 peak activity was only 13% of that determined at pH 5.5. The focusing profile obtained with peak II enzymes showed (Fig. 2a) a major peak, pI 5.27, of hexosaminidase A activity and a minor band of hexosaminidase B activity. As shown (Fig. 2b), components A and C of the original brain extract were not separated in this Ampholine range. Nevertheless the two sides of the focused peak do show different activity pH optima consistent with a partial enzyme separation.

Electrofocusing of hexosaminidase C in the narrow-range Ampholine, pH 4–6, again resulted in a single activity peak of pI 4.80. This isoelectric point, although supporting that expected from the electrophoretic mobility of hexosaminidase C, conflicts with that of pI 5.70 reported by Penton *et al.* (1975). Unfortunately, these authors do not report their own findings on pI values of other forms of hexosaminidase on which to base some comparison.

Since the serum form of hexosaminidase A is also electrophoretically more anodic than the tissue A form (Swallow *et al.*, 1974), it too was focused, to compare its profile with that of hexosaminidase C. In Ampholine range pH 4–6, serum hexosaminidase A had pI 5.00, intermediate between those of tissue hexosaminidases A and C. Serum hexosaminidase A is neuraminidase-sensitive (Swallow *et al.*, 1974; Ikonne & Ellis, 1973), and it seems likely that much of its net negative charge is provided by bound sialic acid residues. Such a situation seems unlikely to be also true for hexosaminidase C, despite its low pI, since, unlike serum hexosaminidase A, the C isoenzyme was not found to shift to a higher pI after treatment with bacterial neuraminidase (G. T. N. Besley, unpublished work).

The properties of hexosaminidase C with respect to its molecular weight, activity pH optimum, substrate specificity and isoelectric point clearly indicate a molecular form distinct from hexosaminidases A and B. It seems likely, then, that hexosaminidase C does not share common subunit structures with

Fig. 1. Sephadex G-200 gel filtration of human brain extract

The column (90 cm $\times$ 3.3 cm diam.) of Sephadex G-200 was eluted at a flow rate of 24 ml/h with 10 mM-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl, 0.1% (v/v) Triton X-100 and 0.02% (w/v) NaN₃. The run was performed at 4°C and 8.1 ml fractions were collected. Hexosaminidase activity was measured at pH 4.5 (○) and pH 5.5 (●).

Fig. 2. Isoelectric-focusing profiles of human brain hexosaminidases

Focusing was performed over 18 h at 4°C and at 400 V in an Ampholine gradient in the range pH 3.5–10. (a) Hexosaminidases A and B (○) (0.2 ml from frozen-stored peak II preparation) were electrofocused and assayed at pH 4.5; hexosaminidase C (●) (0.2 ml from frozen-stored peak I preparation) was assayed at pH 5.5. (b) Total brain extract. Frozen-stored brain was homogenized in 12.5 vol. of water and centrifuged at 1000g for 10 min, and the supernatant fraction (0.1 ml) was electro-focused. Hexosaminidase activity was determined at pH 4.5 (○) and pH 5.5 (●).

forms A or B (Ropers & Schwantes, 1973; Galjaard *et al.*, 1974), but is under separate genetic control. This being so, the C form should be retained in GM₂-gangliosidosis; this indeed seems to be the case (Broadhead & Besley, 1975; Penton *et al.*, 1975). In further support of this, the major residual hexosaminidase component of Sandhoff brain has also been found (G. T. N. Besley, unpublished work) to possess an activity pH optimum, substrate specificity and pI

identical with those described above for normal brain hexosaminidase C. However, hexosaminidase C activity is highly labile, and when activity is lost the minor, more stable, acid component, hexosaminidase S, may be revealed.

This work was supported in part by the Scottish Advisory Committee on Medical Research and the Secretary of State for Scotland.

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