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HEXOSAMINIDASE C IN TAY-SACHS AND SANDHOFF DISEASE

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Summary

1. Hexosaminidase C has been purified from human placenta. Complete separation from hexosaminidases A and B was achieved.

2. The following properties of hexosaminidase C differ from those of the A and B isozymes. Presence in the supernatant rather than the lysosomes, neutral pH optimum, higher molecular weight, lack of activity on β-*N*-acetylgalactosamine derivatives, and lack of immunological relationship.

3. Hexosaminidase C is active in patients deficient in hexosaminidases A and B and can be recognized by its characteristic electrophoretic mobility. It is concluded that the genetic origin of hexosaminidase C is probably different from that of hexosaminidases A and B.

Introduction

Hexosaminidase C was first described in human brain by Hooghwinkel et al. [1]. Its presence was demonstrated in all human tissues by Poenaru and Dreyfus [2]. It may be related to a β-*N*-acetylglucosaminidase described previously by Frohwein and Gatt in the cytoplasm of calf brain [3—4]: Preliminary studies on physicochemical and immunological properties of hexosaminidase C [5—6] showed clear differences with the previously known isozymes, hexosaminidases A and B. In the present work we show the persistence of hexosaminidase C in Tay-Sachs' and Sandhoff diseases. In addition, a more detailed study of the properties of hexosaminidase C was carried out and its purification was undertaken.

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Materials and methods

Materials

The enzymatic source was human placenta, used as soon as possible after delivery. White blood cells from control and hexosaminidase-deficient patients were prepared by dextran sedimentation followed by hypotonic hemolysis.

Methods

Hexosaminidase assay. The assay used as a substrate a derivative of 4-methyl-umbelliferone. To 100 μl of 4-methyl-umbelliferyl 2-acetamido-2 deoxy β-D-glucopyranoside 10^{-3} M citrate/phosphate buffer pH 4.4 were added 20 μl of enzyme solution. After 1 h at 37° C the reaction was stopped by adding 3 ml of molar glycine buffer, pH 10. The fluorescence of the alkaline methylumbelliferone was estimated with an Aminco fluorimeter, set at wavelengths of 366 nm (excitation) and 446 nm (emission).

Thermal inactivation took place at 50° C for 3 h according to O'Brien et al. [7].

Antisera were obtained in rabbits against preparations of hexosaminidases. Hexosaminidase A was purified as described previously. Hexosaminidase B was purified from human placenta by the following procedure: Lysosomes were isolated by centrifuging a placenta homogenized in 0.25 M sucrose for 10 min at 800 × *g* and the supernatant for 20 min at 18 000 × *g*. The supernatant with a specific activity of 5.1 μM/mg protein/min (total hexosaminidase), was poured through a DE_{52} column. The fraction containing the hexosaminidase B free of hexosaminidases A and C was precipitated by 60% saturated $(NH_4)_2SO_4$ and after dialysis against isotonic saline, was used to immunize rabbits. Immunization was obtained by injecting three times solutions containing approx. 1 mg of protein (with a specific activity of 340 μM/mg protein/min) emulsified with complete Freund adjuvant in multiple intradermal injections.

Electrophoresis of hexosaminidases was performed as described previously [2] on cellulose acetate strips (Cellogel) in 0.04 M phosphate buffer, pH 6.5, followed by incubation with the substrate. Addition of alkali at the end of the incubation period resulted in the appearance of fluorescent bands of 4-methyl-umbelliferone, locating the position of the enzyme, which were photographed.

This incubation took place generally at pH 4.5 or 5.0, which is the usual pH for hexosaminidases A and B. In some experiments, incubations were performed at pH 6.5.

The purification steps are described below.

Results

1. Intracellular localization of hexosaminidase C

After homogenizing and extracting the placenta with 0.25 M sucrose, it could be shown by electrophoresis that hexosaminidase C was absent from the mitochondrial-lysosomal and microsomal fractions, and was found only in the supernatant. The lysosomal fraction contained the major part of hexosaminidase A and B, but a noticeable part was solubilized during homogenization (Fig. 1).

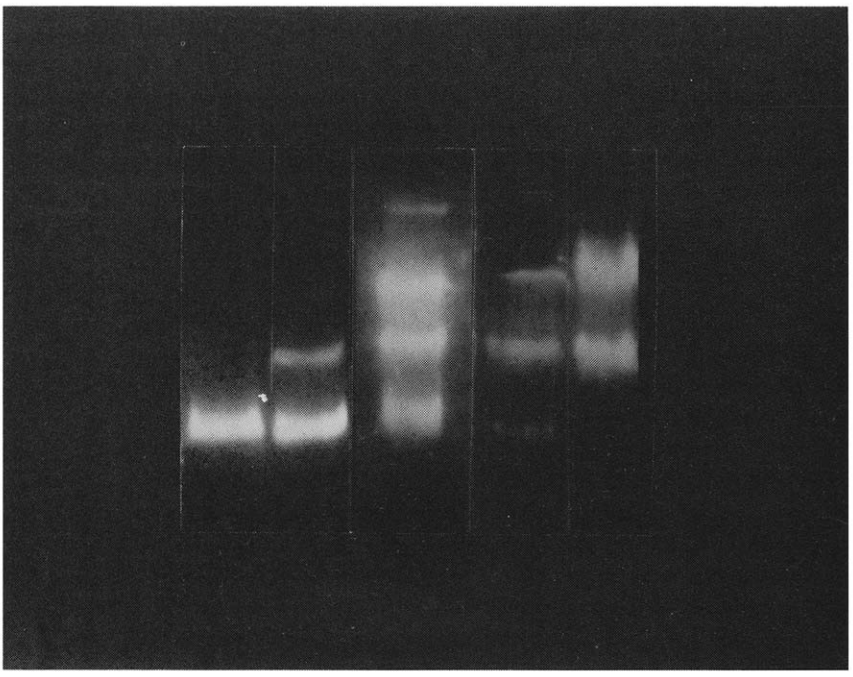


Fig. 1. Electrophoretic separation on cellulose acetate of placenta hexosaminidases during purification. 1, extract of placental lysosomes; 2, supernatant after centrifugation at 18 000 × *g*; 3, number 2 precipitate at 40% $(NH_4)_2SO_4$; 4, number 3 after CM Sephadex; 5, number 4 after Sephadex G-200.

2. Effect of antisera against hexosaminidases A and B

In a previous work [5] it was shown that an anti-hexosaminidase A antiserum does not inhibit hexosaminidase C. The antiserum prepared against hexosaminidase B is also without effect (Fig. 2).

3. Purification of hexosaminidase C

a. Extraction. The placenta washed with saline was cut and homogenized in a Waring Blendor for 30 s in two volumes (W/V) of a 0.01 M Tris · HCl buffer, pH 7.0, containing sucrose 0.25 M and 0.001 M EDTA. After a preliminary centrifugation for 10 min at 800 × *g*, the supernatant was centrifuged at 18 000 × *g* for 30 min to eliminate the lysosomes.

b. Precipitation with ammonium sulphate. The supernatant was brought to 30% saturation with ammonium sulphate. The precipitate was discarded and the supernatant was brought to 40% saturation. After standing for 2 h the mixture was centrifuged for 15 min at 15 000 × *g*. The precipitate contained the whole of hexosaminidase C and still the major part of hexosaminidase A and B. It was dialysed overnight against a 0.01 M phosphate buffer, pH 6.0, containing 0.001 EDTA.

c. Chromatographic steps. The solution was poured through a CM Sephadex column (40 × 2.5 cm) equilibrated with the same buffer. Hexosaminidase B was retained on the column while hexosaminidases A and C were not adsorbed. The effluent was then passed on a column of Sephadex G-200 (85 ×

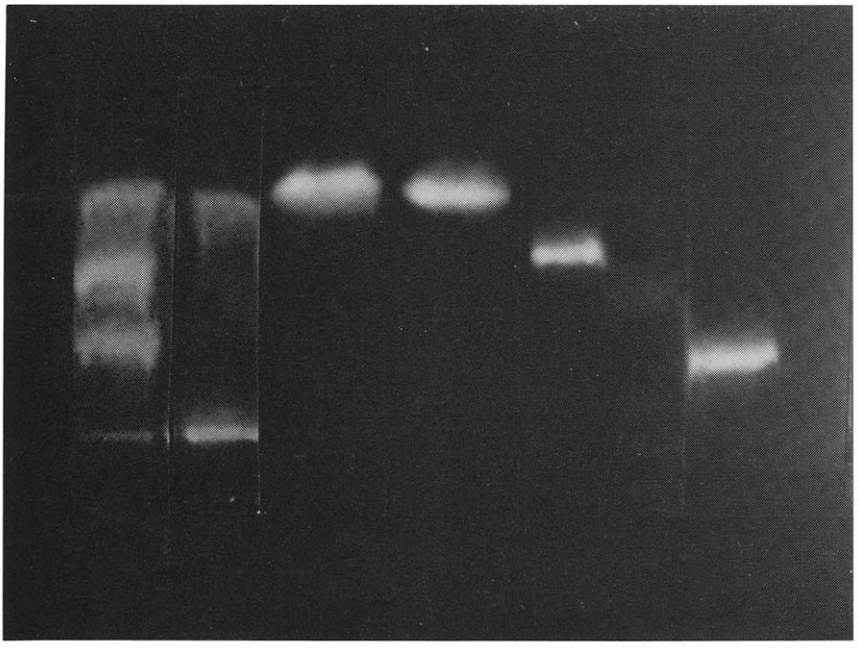


Fig. 2. β-Glucosaminidase electrophoresis. 1, placental extract precipitated at 40% $(NH_4)_2SO_4$; 2, 1 + anti B; 3, hexosaminidase C; 4, hexosaminidase C + anti B; 5, hexosaminidase A; 6, hexosaminidase A + anti B; 7, hexosaminidase B; 8, hexosaminidase B + anti B. Antigen and antibody were mixed and left for 3 h at room temperature and the mixture was centrifuged 10 min at 5000 × *g* before electrophoresis.

2.5 cm) at 6.5 ml/h flow rate. This step afforded separation of hexosaminidase C from hexosaminidase A.

Attempts at purifying further the enzyme on DEAE or DEAE Sephadex columns were unsuccessful. No active hexosaminidase C could be recovered from the columns.

The estimation of the purification of hexosaminidase C was difficult since hexosaminidase C accounted only for a low percentage (2.5%) of total hexosaminidase activity in the initial extract. To determine the activity due to hexosaminidase C in the crude extract an aliquot was treated with an excess of anti-hexosaminidase A antiserum. The residual activity is due to hexosaminidase C alone. The purification obtained (hexosaminidase C activity per mg protein) was about 20 times, with an overall yield of 40%. The purification steps are summarized in Table I. The electrophoretic picture after each step is shown in Fig. 1.

4. Properties of hexosaminidase C

a. The size of hexosaminidase C. This was estimated on Sephadex G-200 by comparing with the following proteins : Rabbit muscle phosphorylase *b* (mol. wt 185 000) [8], aldolase (150 000) [9], hexosaminidase A (130 000) [10]. None of the proteins appeared in the void volume (20 first tubes). Elution peaks of phosphorylase and hexosaminidase C were found at tube 50, of aldolase at tube 65, of hexosaminidase A at tube 75. The molecular weight

TABLE I

HEXOSAMINIDASE C PURIFICATION STEPS

S_1, supernatant after 800 X g; S_2, supernatant after 18 000 X g; P_3, Precipitate of S_2 at 40% $(NH_4)_2SO_4$; E_1, CM Sephadex column effluent; E_2, Sephadex G-200 column effluent.

Stage	Volume (ml)	Total activity * (nM/min)	Total proteins (mg)	Specific activity (nM/mg protein/min)	Degree of purification	Yield
S_1	790	4 400	11 200	0.39	—	100
S_2	770	4 350	9 600	0.45	1.2	99
P_3	75	2 300	1 600	1.44	3.7	52
E_1	86	2 100	510	4.12	10.5	47
E_2	30	1 900	270	7.03	18	43

* The assays of hexosaminidase C were carried out in the presence of antiserum anti-hexosaminidase A which precipitates the hexosaminidase A and hexosaminidase B. The precipitates were removed by centrifugation before determination.

of hexosaminidase C can be estimated to be approx. 190 000, definitively higher than that of hexosaminidases A and B.

b. Isoelectric point. It was estimated by isoelectric focusing in a pH gradient 3.5—10 according to Drysdale et al. [11] on polyacrylamide gels with direct staining on the gel. A p*I* of 5.7 was found, while those of hexosaminidase A and B are 5.0 and 7.3 [12,13].

c. Activity as a function of pH. The optimal pH for hexosaminidase C differs widely from those of hexosaminidases A and B. The latter, like most lysosomal enzymes, display a maximum of activity around pH 4.5. Optimum for hexosaminidase C is at pH 7 (Fig. 3).

d. Michaelis constant. The K_m has been measured for the artificial substrate, 4 methyl-umbelliferone hexosaminide, at two pH values, e.g. 4.5 and

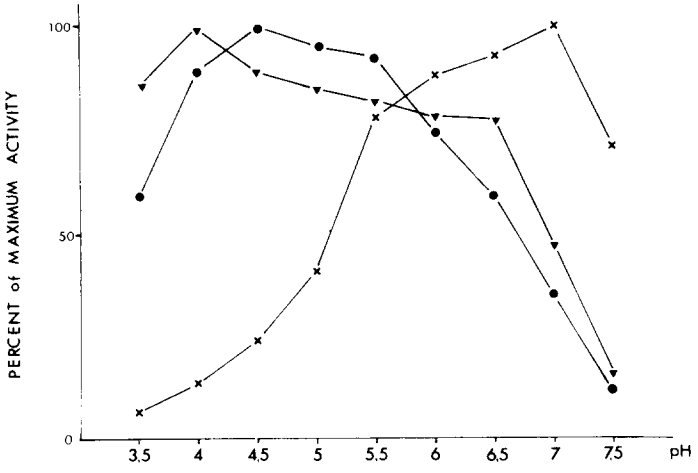
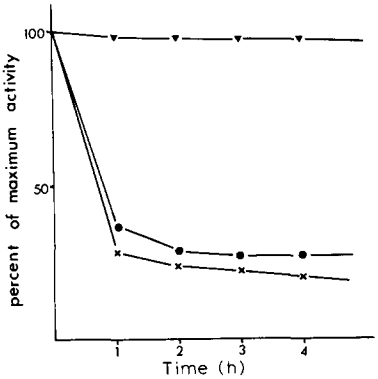


Fig. 3. Hexosaminidases activities according to pH, expressed as per cent of activity at pH optimum. ▼———▼, hexosaminidase B; ●———●, hexosaminidase A; X———X, hexosaminidase C.

7.0, to compare with hexosaminidases A and B. The K_m at both pH values was $8.3 \cdot 10^{-4}$. The K_m of hexosaminidases A and B was $13 \cdot 10^{-4}$ M. This difference cannot be regarded as significant.

e. Relative activity on glucosamine and galactosamine derivatives. The assays on the β-*N*-acetyl galactosamine derivative, taking the glucosamine derivative at pH 4.5 as 100 p 100 were performed at pH 4.5 and 7.0. At pH 4.4, the activity of hexosaminidase C on galactosaminide was 4.5 p 100 of that on glucosaminide rather than 20 p 100 for hexosaminidases A and B. At pH 7 it was 2.4 p 100 rather than 8 p 100 for hexosaminidases A and B. The results



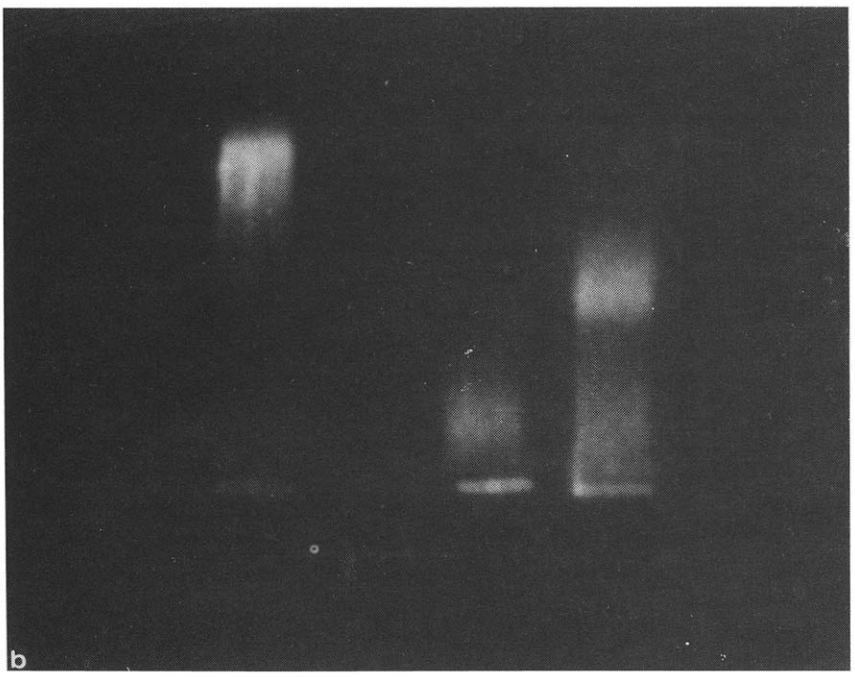


Fig. 4. a) Thermal inactivation curves at 50° C at pH 4.5. ▼———▼, hexosaminidase B; ●———●, hexosaminidase A; X———X, hexosaminidase C. b) Electrophoretic patterns after thermal inactivation at 50° C and pH 4.5. 1. hexosaminidase C; 2. hexosaminidase C after 4 h at 50° C; 3. leucocytic extract after 4 h at 50° C; 4. leucocytic extract.

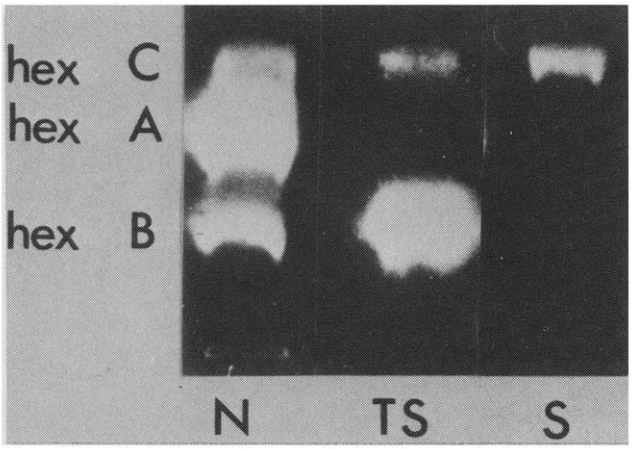


Fig. 5. Electrophoretic separation of extracted from white blood cells β-glucosaminidases. N, control; TS, Tay-Sachs disease; S, Sandhoff's disease.

obtained with hexosaminidases A and B were in agreement with those of others [15].

f. Thermostability. Thermostability was determined at pH 4.5 in a 0.04 M citrate/phosphate buffer in preparations containing the same enzymatic activity and similar concentrations in total protein. The same results (disappearance of forms A and C, and persistance of form B) were obtained with crude extracts containing all three isozymes. Fig. 4 shows that the thermostability of hexosaminidase C was the same as that of hexosaminidase A and much lower that of hexosaminidase B. Hexosaminidase C was nearly completely inactivated after 4 h at 50° C and subsequent electrophoresis showed its disappearance, contrasting with the persistence of the hexosaminidase B spot.

5. Hexosaminidase C in patients deficient in hexosaminidase A or B

We had the opportunity of examining white blood cells of six patients with Sandhoff's disease (deficiency in hexosaminidases A and B). In all cases electrophoresis showed the presence of hexosaminidase C, which in most cases looked stronger than in controls. Hexosaminidase C was also present in one case of classical Tay-Sachs disease (deficiency in hexosaminidase A) (Fig. 5).

Discussion

In the present work, we achieved a partial purification of hexosaminidase C. The enzyme was completely freed from isozymes A and B without resorting to the use of antibodies or to affinity chromatography. The purification was limited by the lability of the enzyme which did not stand anion exchangers. The state of purity obtained allowed to investigate a number of physicochemical properties.

Hexosaminidase C is a β-hexosaminidase which differs from the previously known isozymes A and B by numerous properties:

1 Cellular localization in the soluble fraction rather than the lysosomes.

2 Neutral optimum of pH activity.

3 Different isoelectric point and affinity for the substrate.

4 Very low *N*-acetyl-galactosaminidase activity.

5 Larger size of the molecule.

6 Absence of immunological relationship.

The above properties point to a genetic origin which is different for hexosaminidase C from that of hexosaminidase A and hexosaminidase B. This conclusion is supported by the presence of hexosaminidase C in the tissues of patients deficient in hexosaminidases A and B, which we have found in white blood cell extracts from all patients with Sandhoff's disease. In Tay-Sachs' disease, ourselves as well as Vamos (personal communication) have also found the presence of hexosaminidase C, but others [1,16] found it to be absent. Indeed, hexosaminidase C must be looked for only in fresh tissues because it is much more labile than the other forms in stored tissues.

Recently Stirling [14] described the presence of an enzyme, *N*-*N'*-diacetylchitobiase, in tissue extracts of normal subjects and also of patients with Sandhoff's disease, and the question of its relationship to hexosaminidase C has been raised. The two enzymes, however, are probably different, since chitobiase is eluted from Sephadex columns after hexosaminidase A, contrasting with our findings with hexosaminidase C.

Finally, a practical importance of the recognition of hexosaminidase C in human pathology comes from its thermolability, which is analogous to that of hexosaminidase A. In Sandhoff's disease, the residual activity is about 10 p 100 of that of controls. It is entirely destroyed in the heat stability test. While definitely lower than in heterozygotes, this small activity might in some cases be mistaken for a residual activity of hexosaminidase A and lead to a diagnosis of heterozygosity in a homozygous deficient patient. The same error could happen with extracts from cultured amniotic cells. We can conclude therefore, that electrophoresis must be performed in all cases to assess the diagnosis, since it allows a clear separation between the three isozymes. One must remark, however, that hexosaminidase C is not a source of error in plasma and urine, in which it is not present, or in such low amounts that it cannot be found by conventional methods.

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