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STUDIES ON β-D-*N*-ACETYLHEXOSAMINIDASE

VARIOUS ISOZYMES IN TISSUES OF NORMAL SUBJECTS AND SANDHOFF'S DISEASE PATIENTS

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Summary

Hexosaminidase (EC 3.2.1.52) activity from human liver and kidney extract was completely precipitated by anti-hexosaminidase A antiserum and 80 to 90% by anti-hexosaminidase B antiserum. Immunologically distinct hexosaminidase "C" could not be detected in these tissues. The final fractions of hexosaminidase A eluted from DE-52 chromatography were resolved into several enzymatically active components by rechromatography. Compared to hexosaminidase A and B, these minor components are more anodal in polyacrylamide disc electrophoresis. The residual activity of hexosaminidase from liver and fibroblasts of patients with Sandhoff's disease has also been resolved into similar components. The enzyme activity of these more anodal hexosaminidase components was precipitated completely by anti-hexosaminidase A antiserum and partially by anti-hexosaminidase B antiserum. The minor, more anodal components probably represent hexosaminidase molecules having an altered ratio of subunits or the degradation products of hexosaminidase A.

Introduction

Hexosaminidase has been found to exist mainly in two forms, hexosaminidase A and B. Hexosaminidase A is absent in all tissues from patients having Tay-Sachs disease [1—4], whereas both hexosaminidase A and B are absent in tissues from patients having Sandhoff's disease [4,5]. Both, Tay-Sachs and Sandhoff's diseases are autosomally inherited and characterized by generalized GM_2 gangliosidosis. Hexosaminidase A and B have been purified to homogeneity and their structural, kinetic and immunological properties have been

studied [6—10]. Recently, Braidman and co-workers [11,12] have reported that a significant portion of the total hexosaminidase activity in the normal human liver and brain is hexosaminidase "C". Hexosaminidase C is reported to have a pH optimum of between 6 and 7 compared to pH 4.4 optimum of hexosaminidase A and B. These workers have reported that hexosaminidase C is immunologically distinct from hexosaminidase A and B and is present in liver and brain of Tay-Sachs patients [11]. Their studies indicated that the synthesis of hexosaminidase C was controlled by genes other than those coding for the synthesis of hexosaminidase A and B. Using the antiserum raised against the homogeneous preparation of placental hexosaminidase A [8,10], we were able to absorb the entire hexosaminidase activity from normal human liver and brain. This indicated that there is no hexosaminidase component in tissues which is immunologically distinct from hexosaminidase A.

Hexosaminidase from normal human liver, kidney and brain was resolved into at least two or three minor components in addition to A and B by DEAE-cellulose (DE-52) column chromatography. These minor components are more anodal than hexosaminidase A in polyacrylamide disc electrophoresis. The residual enzyme activity in the fibroblasts and liver from Sandhoff's patients was also resolved into three or four components by DE-52 column chromatography. These components from Sandhoff's tissues were similar in chromatographic and immunologic properties to minor components from normal human tissues.

Materials and Methods

4-Methylumbelliferyl β-D-*N*-acetylglucosaminide was purchased from Koch-Light Laboratories, England. Concanavalin A insolubilized on beaded Agarose and CNBr-activated Sepharose 4B were purchased from Sigma Chemical Company. Fibroblast culture of Sandhoff's disease No. GM-294 was obtained from The Human Genetic Mutant Cell Repository Institute for Medical Reserach, Cambden, N.J. Cellogel strips were purchased from Brinkmann Company. All the other reagents were of analytical grade.

Liver and brain tissues from infants and kidneys from adults were obtained after post-mortem and used immediately or stored at −20° C. In the case of kidney, only cortex was used. A 20% homogenate of the tissues was prepared in 10 mM phosphate buffer, pH 6.0, by using Sorvall omnimixer at a speed of 8000 rev./min for 15 min. The homogenates were frozen and thawed two times and stirred at 4° C for 4 h before centrifugation at 32 000 × *g* at 4° C. The supernatants from all the samples except kidney were subjected to DE-52 column chromatography, after dialysis against phosphate buffer, 10 mM, pH 7.5. In the case of kidney, the supernatant was passed through a column of concanavalin A insolubilized on beaded Agarose and hexosaminidase was eluted by α-methylmannoside as described ealier [13]. The enzymatically active fractions were subjected to DE-52 column chromatography. The last fractions of liver and kidney eluted from DE-52 column, marked by arrows (Figs. 1, 2), were subjected to a second DE-52 chromatography. The experimental conditions are described in the respective figure legends. For serological studies, antibodies were raised by injecting homogeneous hexosaminidase A and B from

human placenta into rabbits as described previously [10]. Cellogel electrophoresis was performed as described by Poenaru and Dreyfus [14]. Polyacrylamide disc electrophoresis was carried out according to Davis [15]. Protein was determined by the method of Lowry et al. [16]. Determination of hexosaminidase activity and staining of the gels for enzyme activity was performed as described previously [8].

Results

Attempts to demonstrate the presence of immunologically distinct hexosaminidase C

Cellogel and polyacrylamide disc electrophoresis. Both polyacrylamide disc electrophoresis and cellogel electrophoresis resolved the liver and brain hexosaminidase from the 32 000 × *g* supernatant (as described in Methods) into two bands which corresponded to homogeneous placental hexosaminidase A and B after staining for enzyme activity at pH 4.4 or 7.0. Hexosaminidase from liver and fibroblasts of Tay-Sachs patients migrated as a single band on polyacrylamide disc electrophoresis which corresponded to hexosaminidase B. Identical results were obtained when the tissues were homogenized in the presence of Triton X-100, 0.05%, as reported by Braidman et al. [11]. The Sandhoff's liver and fibroblasts supernatant did not stain for the enzyme activity on polyacrylamide disc electrophoresis.

Serological studies. The supernatant from liver or brain homogenates prepared in phosphate buffer with or without Triton X-100 were incubated with equal amounts of antiserum raised against homogeneous placental hexosaminidase A or hexosaminidase B for 24 h at 4°C. The samples were centrifuged and the supernatant was assayed for hexosaminidase activity at pH 4.4 and 7.0. No enzyme activity was detected in the supernatant of samples incubated with anti-hexosaminidase A antiserum (Table I), whereas, in the samples which were incubated with anti-hexosaminidase B antiserum, about 10% of enzyme activity was detected in the supernatants (Table I). However, the pH optimum of the unabsorbed enzyme activity was found to be 4.4 (Table I). Identical results were obtained when anti-hexosaminidase B antiserum linked to CNBr-activated Sepharose 4B was used to absorb hexosaminidase. Thus, by using a potent antiserum raised against homogeneous hexosaminidase A and B from placenta, we could not show the presence of an immunologically distinct hexosaminidase C which had a pH optimum of 7.

Studies of minor components of hexosaminidase

Hexosaminidase from the fibroblasts of Sandhoff's patient having about 1% of the normal enzyme activity was resolved into two components by DE-52 column chromatography (Fig. 1). One component which was eluted at a salt concentration of about 130 mM NaCl and pH 6.51 corresponded to hexosaminidase A. The other component was eluted at a higher salt concentration, i.e. 175 mM NaCl and pH 6.24. In one of the liver samples from a Sandhoff's patient identical results were obtained. However, in a second sample of liver obtained from another Sandhoff's patient (not related to the first case), at least three enzymatically active components were separated at salt concentration

TABLE I

PRECIPITATION OF HEXOSAMINIDASE FROM THE TISSUES OF NORMAL SUBJECTS AND SANDHOFF'S PATIENTS WITH ANTI-HEXOSAMINIDASE A AND B ANTISERUM

Samples were incubated with anti-hexosaminidase A and B antiserum as described in the text. Since all the enzyme activity of samples incubated with anti-hexosaminidase A antiserum was precipitated, only the results of precipitation of hexosaminidase by anti-hexosaminidase B antiserum are reported in this Table.

Sample	Enzyme activity (munits*/ml)				Inhibition at pH 4.4 (%)
	Without antiserum		With antiserum		
	pH 4.4	pH 7.0	pH 4.4	pH 7.0	
Before DE-52					
Normal liver	85	13	7	1	92
Normal brain	107	32	13	5	88
After DE-52					
Normal liver**	1.42	—	0.47	—	67
Sandhoff's liver	1.75	—	0.3	—	83
Sandhoff's fibroblast	0.88	—	0.12	—	86
Normal kidney rechromatography peaks**					
No. 1	30.87	—	7.56	—	76
No. 2	30.24	—	7.14	—	76
No. 3	24.57	—	7.14	—	71
No. 4	31.50	—	12.60	—	60
No. 5	44.10	—	18.48	—	58
No. 6	34.65	—	13.65	—	61

* One milliunit of enzyme catalyzes the formation of one nmol of *N*-acetylglucosamine from 4-methylumbelliferyl β-D-*N*-acetyl glucosaminide per min at 37°C.

** These are samples from DE-52 rechromatography of late fractions of first DE-52 column as marked by arrows in Figs 1 and 2.

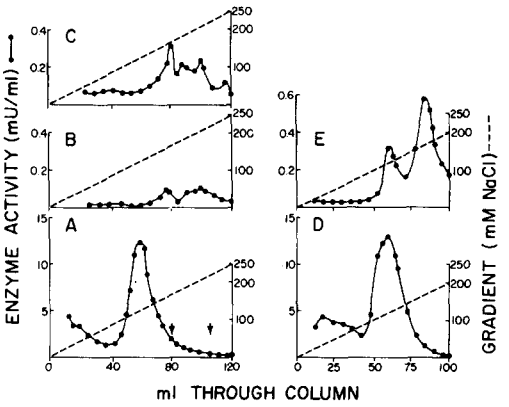

Fig. 1. Separation of hexosaminidase isozymes from normal and Sandhoff's tissues by DEAE-cellulose (DE-52) column chromatography. DE-52 equilibrated with 10 mM phosphate buffer, pH 7.5, was poured into transfer pipettes (Pasteur type) to a bed height of 7 cm. The supernatant fractions (as described in the text) were passed through the column at a rate of 15 ml/h. The enzyme from the column was eluted by a linearly increasing NaCl gradient and a simultaneously decreasing pH gradient from 7.5 to 6.0. A, normal liver; B, rechromatography of samples between the arrows from Fig. 1A; C, Sandhoff's liver; D, normal fibroblasts; and E, Sandhoff's fibroblasts.

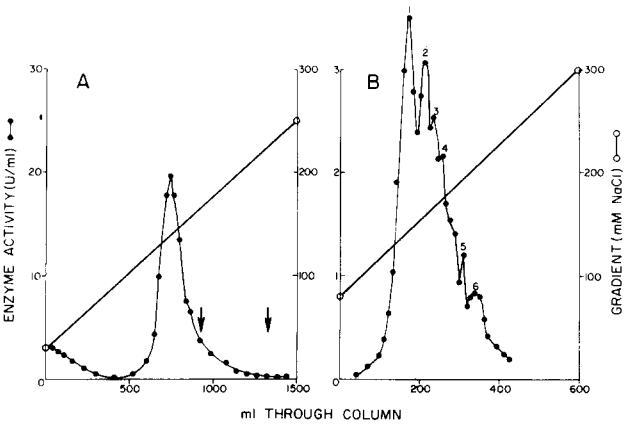

Fig. 2. Separation of hexosaminidase isozymes from human kidney by DEAE-cellulose (DE-52) column chromatography. A. The enzymatically active fractions of kidney hexosaminidase eluted from the concanavalin A column (details will be described elsewhere) were passed through a DE-52 column (41 cm × 2.5 cm) equilibrated with 10 mM phosphate buffer, pH 6.0, at a rate of 50 ml/h. Hexosaminidase A was eluted with a 1500 ml of linearly increasing NaCl gradient from 30 to 250 mM in 10 mM phosphate buffer, pH 6.0. B. Rechromatography of late fractions of kidney hexosaminidase as marked by arrows in Fig. 2A. The enzymatically active fractions were passed through a DE-52 column (30 cm × 2.5 cm) equilibrated with 10 mM phosphate buffer, pH 6.0, at a rate of 30 ml/h. The elution was carried out with a 600 ml of linearly increasing NaCl gradient from 80 to 300 mM in 10 mM phosphate buffer, pH 6.0.

higher than that required for the elution of hexosaminidase A (Fig. 1).

In the first DE-52 column chromatography, normal liver hexosaminidase was resolved into two major isozymes. One isozyme, hexosaminidase B, did not bind to DE-52 whereas, the other isozyme, hexosaminidase A, that was bound to DE-52, was eluted by about 130 mM NaCl (Fig. 1A). On rechromatography, the last eluted fractions of hexosaminidase A (as marked by arrows in Fig. 1A) were resolved into two enzymatically active components. Both components were eluted at higher salt concentration than that required for the elution of hexosaminidase A.

In the purification of hexosaminidase A and B from human kidney cortex, the first step was the absorption of hexosaminidase activity on concanavalin A insolubilized on beaded Agarose column followed by elution with α-methyl-mannoside (details will be published elsewhere) and the second step was DE-52 column chromatography. The elution pattern of hexosaminidase A is presented in Fig. 2A. On rechromatography, the tail fractions (marked by arrows, Fig. 2A) were resolved into at least six components (Fig. 2B) of which five were eluted at salt concentrations higher than that required for the elution of hexosaminidase A as presented in Fig. 2A. On polyacrylamide disc electrophoresis, Peaks 2 to 6 (Fig. 2B) containing hexosaminidase activity were found to be faster moving than hexosaminidase A (Fig. 3). This indicated that these enzyme bands are more negatively charged than hexosaminidase A and that the charge increased from Peaks 2 to 6.

Serological studies. For immunosorption studies, 10-μl samples containing 10 munits of hexosaminidase activity were incubated overnight at 4°C with 10 μl of anti-hexosaminidase A serum or anti-hexosaminidase B serum, 50 μl 20 mM phosphate buffer, pH 7.0, and 130 μl of 0.05% bovine serum albumin. The

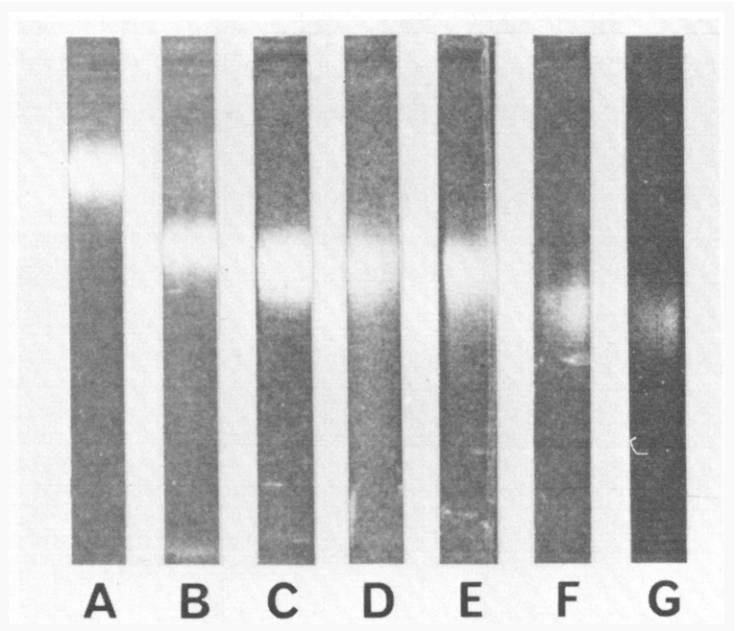

Fig. 3. Separation of hexosaminidase isozymes from normal kidney by polyacrylamide disc electrophoresis. Aliquots from various Peaks 1 to 6 (see Fig. 2B) containing 1 unit/ml hexosaminidase were subjected to polyacrylamide disc electrophoresis using a double buffer system, Tris/glycine, 0.052 M, pH 8.9, in the upper chamber and Tris/HCl, 0.1 M, pH 8.2, in the lower chamber. Staining of the gels for enzyme activity was performed as previously described [6]. A, purified kidney hexosaminidase B. B, purified kidney hexosaminidase A. C, D, E, F and G, hexosaminidase from Peaks 1, 3, 4, 5 and 6, respectively, from Fig. 2B.

samples were centrifuged at 30 000 × *g* for 1 h at 4°C. Aliquots from the supernatant were assayed for enzyme activity. The control samples contained no antiserum.

Anti-hexosaminidase A antiserum completely precipitated the enzyme activity from all the minor components of hexosaminidase isolated from the tissues of normal and Sandhoff's patient. However, anti-hexosaminidase B antiserum precipitated 80 to 90% of hexosaminidase from the enzymatically active components of Sandhoff's liver and fibroblasts. On the other hand, anti-hexosaminidase B serum adsorbed 55 to 75% of the enzyme activity from the enzymatically active minor components of normal liver and kidney.

Various enzymatically active components (Peaks 1 to 6) separated from the rechromatography step of kidney hexosaminidase A tail fractions (Fig. 2B) were subjected to polyacrylamide disc electrophoresis (Fig. 3) and double immunodiffusion studies (Fig. 4). In polyacrylamide disc electrophoresis, the mobility of the enzyme towards anode from Peaks 1 to 6 increased. In double immunodiffusion studies, the fractions from Peaks 1, 2, 3 and 4 gave precipitin lines as presented in Fig. 4. The unprecipitated proteins were extracted with 2 M NaCl containing 0.05 M NaN_3. Since the enzyme-antibody complex is enzymatically active, the gel was stained for enzyme activity. All the precipitin lines were found to be enzymatically active (Fig. 4). Although fraction No. 6 failed to give a visible precipitin line with antiserum, it stained for the enzyme activity (Fig. 4).

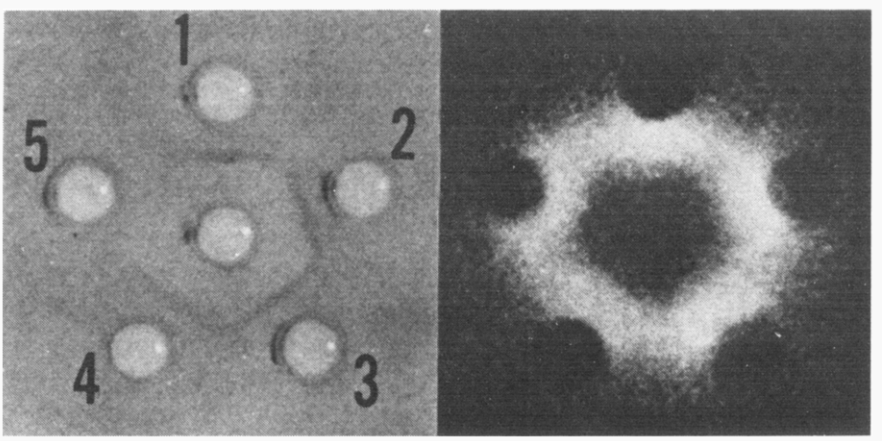

Fig. 4. Agar double immunodiffusion studies using hexosaminidase B antiserum. 8 μl of anti-hexosaminidase B serum was placed in the center well. Aliquots, 8 μl, from the peak Fractions 1, 2, 3, 4 and 6 (as marked in Fig. 2B) were placed in outer wells 1 to 5, respectively. Unprecipitated proteins were extracted with 2 M NaCl and stained for enzyme activity. The left and right figures represent before and after staining for enzyme activity, respectively.

Discussion

Two major isozymes of hexosaminidase, namely, A and B, have been found in various human tissues. Hooghwinkel et al. [17] first demonstrated the presence of an electrophoretically more anodal hexosaminidase component than the A isoenzyme and designated it as hexosaminidase C. This component was found to be present in human brain and absent in human liver. However, Poenaru and Dreyfus [14] were able to separate this minor component on cellogel electrophoresis in both human liver and brain. Braidman and coworkers [11,12] have also recently reported the presence of hexosaminidase C in various human tissues. They have shown that hexosaminidase C is more electronegative than hexosaminidase A and has a pH optimum of about 7.0 as compared to 4.4 for hexosaminidase A and B. They have also shown that antisera raised against partially purified hexosaminidase A or B failed to absorb hexosaminidase C. Based on these observations, a separate genetic control for hexosaminidase C has been suggested [12].

We have investigated the presence of hexosaminidase C in various human tissues using antisera raised against homogeneous placental hexosaminidase A and B. Complete absorption of hexosaminidase activity by anti-hexosaminidase A antiserum indicated that hexosaminidase C is not immunologically distinct from hexosaminidase A and thus is not under a separate genetic control as suggested by Braidman et al. [11,12]. Whereas, the antisera raised against pure hexosaminidase A completely absorbed the hexosaminidase activity from normal human tissues, hexosaminidase B antisera absorbed only 80 to 90% of enzyme activity. However, the pH optimum of residual (unabsorbed) hexosaminidase activity was 4.4 and the activity at 7.0 was only about one-seventh of the activity at pH 4.4. This clearly indicates the absence of any hexosaminidase C type enzyme in this fraction. Partial absorption of hexosaminidase from human tissues by anti-hexosaminidase A antiserum as observed by previous

workers could be due to a weak antiserum raised against impure hexosaminidase.

Liver and fibroblasts from Sandhoff's patients were found to have 1% of total hexosaminidase activity present in normal tissues. Elution of this enzyme from DE-52 column at salt concentrations higher than that required for the elution of hexosaminidase A indicated the enzyme from Sandhoff's tissues has a higher electronegative character. However, the minor components of hexosaminidase (besides A and B) from normal liver and fibroblasts which represent less than 1% of total activity of hexosaminidase in respective tissues were found to have chromatographic and immunological properties similar to the enzyme present in Sandhoff's tissues. These minor components of hexosaminidase present in normal tissues which require a higher salt concentration for elution than hexosaminidase A on DE-52 chromatography were further purified by a second DE-52 column chromatography (Fig. 1) and were found to be more anodal than hexosaminidase A on polyacrylamide disc electrophoresis (Fig. 1). A similar elution pattern of hexosaminidase from DE-52 column and similar serological properties of hexosaminidase from Sandhoff's tissues to the minor hexosaminidase components of normal tissues suggests that the residual hexosaminidase activity in Sandhoff's disease patients is similar to the minor hexosaminidase components of normal tissues. Thus, the enzyme in the tissues of Sandhoff's disease designated by Ikonne and Desnick [18] as hexosaminidase S does not appear to be a unique isozyme having a separate genetic origin from hexosaminidase A and B.

We have demonstrated earlier that both hexosaminidase A and B are hexamers [8]. Based on structural and immunological studies, we have proposed that hexosaminidase A and B share a common subunit (β) and that hexosaminidase A has a unique subunit (α) [7,10,19]. Thus, hexosaminidase A is probably $(\alpha\beta)_3$ and hexosaminidase B is $(\beta\beta)_3$ [7,10]. Carroll and Robinson [20,21] have also suggested that hexosaminidase A and B share a common subunit. This model is further supported by somatic cell hybridization studies [22,23]. According to this model Tay-Sachs disease would then be an "α" gene mutation leading to a loss of hexosaminidase A activity and an increase in hexosaminidase B activity due to increased number of β-subunits. Similarly in Sandhoff's disease, the mutation of structural genes coding for the synthesis of β-subunits would lead to an increase in α-subunits. This would then result in an increased ratio of α- to β-subunits in hexosaminidase A.

We, therefore, propose that the minor components of hexosaminidase from normal and Sandhoff's tissues contain both α- and β-subunits but in an increased ratio of α to β than that present in hexosaminidase A. This would explain the increased negative charge found in these minor components. This would also account for a better reactivity of these components with anti-hexosaminidase A antiserum than with anti-hexosaminidase B antiserum. Since the residual enzyme present in Sandhoff's liver and fibroblasts is precipitated by anti-hexosaminidase B antiserum, the enzyme does not appear to be all α-subunit, i.e. $(\alpha)_6$. The possibility of these minor components representing degradation products of hexosaminidase A or the artifacts formed due to glycoprotein-glycolipid interaction as reported by Kint [24] for β-galactosidase cannot be ruled out.

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