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CLEAVAGE OF THE (1→3)-2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL LINKAGE PRESENT IN KERATAN SULFATE. THE A AND B ISOENZYMES OF HUMAN LIVER HEXOSAMINIDASE (EC 3.2.1.30)

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ABSTRACT

The disaccharide 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-^{3}H]-galactitol, prepared from keratan sulfate, was rapidly hydrolyzed by the A and B isoenzymes of normal human liver hexosaminidase (EC 3.2.1.30), and by the B isoenzyme prepared from the liver of a patient who had died of Tay–Sachs disease. The disaccharide substrate was also hydrolyzed by extracts of normal, cultured-skin fibroblasts, and fibroblasts of patients with Tay–Sachs disease, whereas it was not hydrolyzed by fibroblast extracts of patients with Sandhoff disease. Thus, defective degradation of keratan sulfate, secondary to a defect of the β subunits present in the A and B isoenzymes of hexosaminidase, may contribute to the appearance of skeletal lesions in patients affected by Sandhoff disease.

INTRODUCTION

In a previous communication[1], we have described (*i*) the preparation of the disaccharide 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-galactose from bovine-cornea keratan sulfate; (*ii*) its reduction with sodium borotritide, to give the corresponding [1-^{3}H]galactitol; and (*iii*) a method for the measurement of the enzyme β-D-(1→3)-*N*-acetylglucosaminidase using as a substrate the radioactive galactitol disaccharide. In this communication, we have studied the properties of enzymes obtained from human liver, in order to investigate their possible role in the degradation of keratan sulfate.

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A review of the literature indicates that the A and B isoenzymes of hexosaminidase (EC 3.2.1.30), and the minor component hexosaminidase S, are active on a variety of oligosaccharides and glycosaminoglycans containing 2-acetamido-2-deoxy-β-D-galactopyranosyl or -D-glucopyranosyl groups at the nonreducing end[2-4]. Thus, hexosaminidase A cleaves 2-acetamido-2-deoxy-D-galactose from a heptasaccharide derived from chondroitin sulfate[5], and 2-acetamido-2-deoxy-D-glucose or -D-galactose from trisaccharides prepared from hyaluronic acid or desulfated chondroitin 4-sulfate, both having a hexuronic acid residue in subterminal position[6,7]. Moreover, this isoenzyme removes terminal, nonreducing 2-acetamido-2-deoxy-β-D-glucopyranosyl groups from polymeric hyaluronic acid[8] and 2-acetamido-2-deoxy-β-D-galactopyranosyl groups from polymeric chondroitin sulfates and possibly from dermatan sulfate[9].

Information is also available on the activity of hexosaminidase B on various oligosaccharides and glycosaminoglycans. Whereas patients affected by Tay-Sachs disease have a modest accumulation of oligosaccharides, glycopeptides, and glycosaminoglycans[10,11], those affected by Sandhoff disease accumulate substantial amounts of glycopeptides or glycosaminoglycans having a 2-acetamido-2-deoxy-β-D-hexopyranosyl terminal group[9,12,13]. Cantz and Kresse[9] demonstrated, in Sandhoff fibroblasts, an excessive intracellular accumulation of glycosaminoglycans, consisting of chondroitin sulfates and possibly dermatan sulfate. This accumulation, which is secondary to the absence of both hexosaminidases A and B, could be corrected by an extract of Tay–Sachs fibroblasts containing only the B isoenzyme. On the other hand, Bach and Geiger[8] demonstrated that hexosaminidase B also cleaves terminal, nonreducing 2-acetamido-2-deoxy-β-D-glucopyranosyl groups from polymeric hyaluronic acid, at a rate, however, approximately one-third of that of hexosaminidase A. All these data suggest that both A and B isoenzymes are involved in the cleavage of 2-acetamido-2-deoxy-β-D-glucopyranosyl residues present in various glycans.

Keratan sulfate is another glycosaminoglycan containing β-D-linked-2-acetamido-2-deoxy-D-glucose; thus, it is possible that the isoenzymes of hexosaminidase might participate in its degradation. In this paper, we describe a partial purification from human liver of the enzyme β-D-(1→3)-*N*-acetylglucosaminidase which cleaves the disaccharide 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-^{3}H]galactitol prepared from keratan sulfate. We compared the properties of this enzyme with those of the A and B isoenzymes of human hexosaminidase, prepared from a normal liver and from the liver of a patient who had died of Tay–Sachs disease. Moreover, we measured, with the just-mentioned substrate, the β-D-(1→3)-*N*-acetylglucosaminidase activity of fibroblast extracts obtained from normal individuals and from patients with Tay–Sachs and with Sandhoff disease. The results of our studies indicate that the A and B isoenzymes of hexosaminidase possess β-D-(1→3)-*N*-acetylglucosaminidase activity as measured with the aforementioned substrate. These findings suggest that the skeletal lesions occurring during the course of Sandhoff disease are very likely due to a defective degradation of keratan sulfate, secondary to the defect of the A and B isoenzymes of hexosaminidase.

EXPERIMENTAL

Culture and homogenization of human fibroblasts. — Normal and mutant, human-skin fibroblasts were cultured in minimal essential medium (Gibco, Grand Island, NY 14072) containing 10% of fetal-calf serum. Some of the cell lines were from our own collection. Others (GM38, normal; GM77, GM221, GM2968, GM502, Tay–Sachs disease; and GM470, GM203, GM294, GM2094, GM317, Sandhoff disease) were obtained from The Human Cell Repository (Camden, NJ 08103). Confluent monolayers were trypsinized at room temperature with 0.05% trypsin (Gibco)–0.02% sodium ethylenediaminetetraacetate in 0.15M sodium chloride, for 10 min at 37°. The detached fibroblasts were suspended in 0.15M sodium chloride and homogenized by sonication (Sonifier Model W200-R, Heat Systems–Ultrasonics, Plainview, NY 11803), with a microprobe at setting 5, for 20 s at 4°. After centrifugation of the homogenate at 20 000*g* for 10 min at 4°, aliquots of the clear supernatants were used for enzyme assays and protein measurement[14,15].

Enzyme measurements. — β-D-(1→3)-*N*-Acetylglucosaminidase was measured by the method described previously[1], which uses as a substrate 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-^{3}H]galactitol and a McIlvaine citric acid–phosphate buffer of pH 4.4 rather than 3.7. A better separation of unhydrolyzed substrate from the product was achieved by use of ITLC silica gel sheets, No. 61886 (Gelman Sciences, Inc. Instrument Co., Ann Arbor, MI 48106), rather than Eastman silica gel sheets. One unit of enzyme activity is defined as the amount of enzyme that hydrolyses 1% of the substrate·h^{-1}. Hexosaminidase (2-acetamido-2-deoxy-β-D-glucoside 2-acetamido-2-deoxy-β-D-glucohydrolase, EC 3.2.1.30) was measured by the method of Braidman *et al.*[16], using 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Koch–Light Laboratories, Research Products International Corp., Elk Grove Village, IL 60007) as the substrate. The results are expressed as nmoles of product liberated·h^{-1}·mg of protein^{-1}. The protein content of column effluents was monitored for absorption at 280 nm.

Partial purification of β-D-(1→3)-N-acetylglucosaminidase from human liver. — Concanavalin A covalently linked to Sepharose 4B (ConA–Sepharose) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ 08854) and *O*-diethylaminoethyl-cellulose (DE-52) from Whatman, Inc. (Clifton, NJ 07014). Normal human liver, obtained at autopsy was immediately frozen at −20°. Upon thawing, all the operations were performed at 4°. The tissue (257 g wet-weight) was sliced, repeatedly rinsed with cold water to remove blood, and suspended in 0.1M sodium phosphate buffer (5 vol.) (pH 6.8) containing 10mM 2-mercaptoethanol. Homogenization was performed in a commercial Waring Blonder Model CB-5, for 3 min at high speed. The homogenate was centrifuged at 13200*g* for 30 min, and the clear supernatant (1250 mL, containing 23.9 g of protein) was collected. A sample was used to measure the enzyme activity in the pH range 3.5–7.3. To the remainder, solid ammonium sulfate was added to 65% saturation, and the solution was stirred overnight. The precipitate collected by centrifugation at 20 000*g* for 20 min was resuspended in

phosphate buffer (400 mL) and dialyzed for 48 h against four changes (5 L each) of the buffer. Insoluble material present in the retentate was removed by centrifugation at 20 000*g* for 20 min, and the clear supernatant (630 mL, containing 15.14 g of protein) was applied to a conA–Sepharose column (0.9 × 45 cm), packed and equilibrated with the phosphate buffer containing 0.5M sodium chloride[17]. The column was washed with the buffer until the absorbance of the effluent measured at 280 nm decreased to zero; thereafter, it was eluted with the same buffer containing, in addition to the sodium chloride, 0.3M methyl α-D-mannopyranoside. The material thus eluted from the column was precipitated with ammonium sulfate at 65% saturation, and the precipitate obtained was collected and dialyzed as described. The final supernatant (55 mL, containing 300 mg of protein) was applied to a column (1.5 × 28 cm) of DE-52, packed and equilibrated with the phosphate buffer. The column was washed with the buffer until the absorbance of the effluent measured at 280 nm decreased to zero; thereafter, it was eluted with a linear gradient of sodium chloride achieved by mixing 250 mL of 0.1M phosphate buffer with an equal volume of the same buffer containing 0.4M sodium chloride[16]. Fractions (5 mL) were collected and samples of each were used to measure absorption at 280 nm. Representative protein-containing fractions were dialyzed against water, lyophilized, and dissolved in citrate–phosphate buffer (5 mL, pH 4.4). Samples (15 μL) were used for enzyme measurements. Samples of protein fractions having the highest β-D-(1→3)-*N*-acetylglucosaminidase activity were dialyzed again, lyophilized, and used for measurement of enzyme activity in the pH range 3–8. Additional aliquots of the same protein-fractions were dissolved in McIlvaine citric acid–phosphate buffer (pH 4.4) and used for determination of the respective apparent K_m. Essentially the same technique, on a smaller scale, was followed for the preparation of the enzyme from a human liver, obtained at autopsy of a patient who died of Tay–Sachs disease.

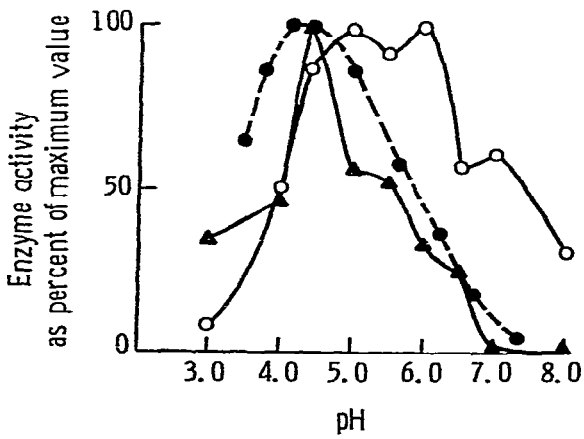

Fig. 1. pH–activity profiles of crude β-D-(1→3)-*N*-acetylglucosaminidase from human liver (●----●), and of purified A (○———○) and B (▲———▲) isoenzymes. Activities were measured in a range of citric acid–phosphate buffers between pH 3.5 and 8.0, with 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-^{3}H]galactitol at a final concentration of 5.7 μM = 10 000 c.p.m.

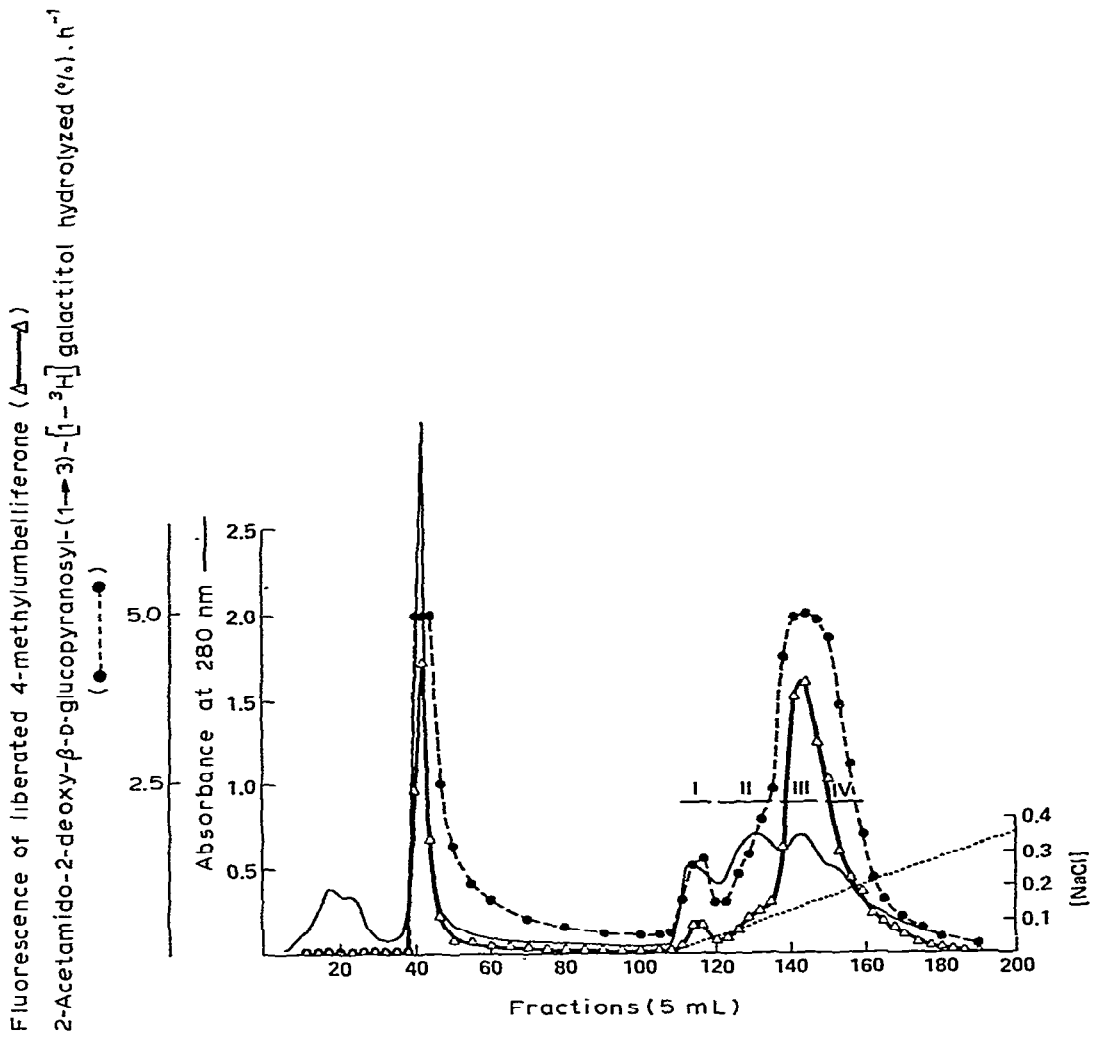

Fig. 2. Chromatography on DE-52 column (1.5 × 28 cm) of a crude, human liver extract, partially purified by ammonium sulfate precipitation and chromatography on conA–Sepharose. The column was packed with 0.1M sodium phosphate buffer (pH 6.8). After application of the sample, the column was washed with buffer (500 mL), and then eluted with a sodium chloride gradient (0–0.4M). The absorbance of the emerging protein was measured at 280 nm. The enzymic activity of samples (15 μL) of representative fractions was measured with 5.7 μM 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-³H]galactitol, (●-----●) and with 0.35 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Δ——Δ). In several fractions, the radioactive substrate was completely degraded during incubation.

RESULTS

The activity of the crude β-D-(1→3)-*N*-acetylglucosaminidase from human liver was measured in McIlvaine 50mM citric acid–phosphate buffers in the pH range 3.5–7.3 (Fig. 1). The enzyme gave a pH–activity curve with a maximum between pH

4.2 and 4.6. For this reason, it was decided to perform all the assays of the enzyme at pH 4.4, rather than at 3.7 as previously suggested.

The material retained on the conA–Sepharose column, and eluted from it with methyl α-D-mannopyranoside, was further chromatographed on anion-exchange cellulose (DE-52). Two major protein-peaks were detected in the void volume: the second (Fig. 2) of these (hexosaminidase B) coincided with peaks of both β-D-(1→3)-*N*-acetylglucosaminidase and hexosaminidase activities measured with the respective substrates mentioned in the Experimental section. With our radioactive substrate, the enzyme had a pH–activity curve similar to that of the crude preparation (Fig. 1) and had an apparent K_m of 0.16mM. An additional group of proteins, eluted by the salt gradient, from its inception to its completion, was arbitrarily separated into four fractions, indicated in Fig. 2 as Fractions I, II, III, and IV. Each one of these fractions had enzyme activities with both substrates (hexosaminidase A). When tested with our radioactive substrate, the pooled fraction III had maximal activity at a pH between 5.0 and 6.0 (Fig. 1), and had an apparent K_m of 0.1mM at pH 4.4.

Similar protein peaks were obtained from a DE-52 column when an extract of Tay–Sachs liver was chromatographed. The peak eluted with the void volume had activity with both substrates (Table I), whereas the peak retained on the column and eluted with the salt gradient had very little activity toward either substrates.

Measurements of β-D-(1→3)-*N*-acetylglucosaminidase in homogenates of human fibroblasts (Table II) indicated that normal and Tay–Sachs fibroblasts had normal activity, whereas Sandhoff fibroblasts had activities either below or at the lower limits of detection.

TABLE I

ACTIVITY[a] OF HEXOSAMINIDASES A AND B ISOLATED FROM THE LIVER OF A PATIENT SUFFERING FROM TAY–SACHS DISEASE

Hexosaminidase	*Amount of protein (μg)*	*Substrate*[b]	
		I (%)	*II (nmol)*
A	12		91
	88	0.10	
B	7.6		1800
	75	1.80	

[a]The activity was measured by the release of [1-^{3}H]galactitol from 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-^{3}H]galactitol (Substrate I) and of 4-methylumbelliferone from 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Substrate II). Units of activities are defined, respectively, as the percentage released, and as the nmoles of substrate hydrolyzed·h^{-1}·mg of protein^{-1}.
[b]Product formed by 1 mg of enzyme protein.

TABLE II

ACTIVITY[a] OF HOMOGENATES OF NORMAL, HUMAN-SKIN FIBROBLASTS AND FIBROBLASTS DERIVED FROM PATIENTS SUFFERING FROM TAY–SACHS DISEASE AND FROM SANDHOFF DISEASE

Fibroblasts	*Protein incubated (μg)*	*Units·h^{-1}·mg^{-1} of protein*
Normal		
M.G.	28	35
R.C.	26	51
B.S.	50	55
A.P.	34	88
J.B.	22	44
GM38	17	77
		Mean ± SD 58 ± 20[b]
Tay–Sachs		
GM77	19	64
GM221	26	54
GM502	23	44
GM2968	20	31
		Mean ± SD 48 ± 14[b]
Sandhoff		
GM203	30	4
GM294	31	2
GM470	18	5
GM2094	17	0
GM317	30	0
		Mean ± SD 2.2 ± 2.2[b]

[a]The activity was measured with 2-acetamido-2-deoxy-β-D-glucosyl-(1→3)-D-[1-^{3}H]galactitol at a final concentration of 5.7μM = 10 000 c.p.m. Units of activity are defined as the percentage of substrate hydrolyzed·h^{-1}·mg^{-1} of protein. The amount of protein incubated for each homogenate is indicated. [b]p of difference between normal and Tay–Sachs fibroblasts >0.1; p of difference between normal and Sandhoff, and Tay–Sachs and Sandhoff fibroblasts <0.005.

DISCUSSION

The pH–activity profile of the crude liver extract and of the purified hexosaminidase B, determined with 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-^{3}H]galactitol as the substrate, produced a symmetrical curve identical to that obtained by Braidman *et al.*[16] for liver hexosaminidase B with 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate. Moreover, when the extracts of normal and Tay–Sachs livers were chromatographed on DE-52 anion-exchange cellulose, the major protein fractions were eluted in a pattern reminiscent of that obtained with the A and B isoenzymes of hexosaminidase[16,17]. The protein fractions of the normal liver extracts were assayed for enzyme activity with the two substrates. Both activities were found to be present and coincident with the protein peaks. When the extract of Tay–Sachs liver was chromatographed on a DE-52 cellulose column, the major protein-fractions obtained were assayed for enzymic activity: the B isoenzyme had normal activities with both substrates, whereas the A isoenzyme

had extremely low activities. Thus, the integrity of the $\alpha_2\beta_2$ heteropolymer is required for activity toward both substrates.

The results obtained with homogenates of normal and mutant fibroblasts are in line with these findings. Homogenates of Tay–Sachs fibroblasts have a normal activity with 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-[1-^{3}H]galactitol, because of the presence of a normal B isoenzyme. However, homogenates of Sandhoff fibroblasts have essentially undetectable activity with the same substrate. This is due to the mutation in the structural gene coding for the common polypeptide chain β, which causes both A and B isoenzymes to be inactive. From these data, it may be concluded that the enzymes responsible for the hydrolysis of the 2-acetamido-2-deoxy-β-D-(1→3)-glucopyranosyl residues present in keratan sulfate are the A and B isoenzymes of hexosaminidase (EC 3.2.1.30).

Because both isoenzymes are deficient in Sandhoff disease, it is likely that the skeletal lesions present in the affected patients are secondary to a defective degradation of keratan sulfate. Similar lesions are conspicuously absent in Tay–Sachs disease, because a functional isoenzyme B assures an adequate degradation of keratan sulfate. In view of the great genetic heterogeneity of GM_2-gangliosidoses[18], it remains to be seen whether disease states may exist in which a mutation in the structural gene coding for the β subunits of hexosaminidase might result in mutant enzymes having altered recognition for keratan sulfate oligosaccharides, but not for GM_2-gangliosides and GA_2-globoside. A similar situation has been clearly demonstrated to occur for some β-D-galactosidase mutants[19–23]*.

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*After this paper had been received and accepted for publication, a paper by Ludolph *et al.*[24] arriving at essentially the same conclusions was published (*Editor*).

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