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PURIFICATION AND SOME PROPERTIES OF LIVER AND BRAIN β-*N*-ACETYL-HEXOSAMINIDASE S

M. POTIER, J. TEITELBAUM, S.B. MELANCON and L. DALLAIRE

Section de Génétique Médicale, Centre de Recherche Pédiatrique, Hôpital Sainte-Justine, Université de Montréal, Montréal H3T 1C5, Québec (Canada)

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Summary

β-*N*-Acetyl-hexosaminidase S (2-acetamido-2-deoxy-β-hexoside acetamido-deoxyhexohydrolase, EC 3.2.1.52) was purified from liver and brain of a patient deceased of type O G_{M2} gangliosidosis (Sandhoff's disease). Brain β-*N*-acetyl-hexosaminidase S was further purified by preparative polyacrylamide gel electrophoresis. The pH optimum of the purified liver and brain enzyme was 5.0 and K_m values were 0.8—0.9 mM and 0.3—0.4 mM with 4-methylumbelliferyl-β-D-*N*-acetylglucosamine and β-D-*N*-acetylgalactosaminide derivatives, respectively. β-*N*-Acetyl-hexosaminidase S was thermolabile losing most of its activity after 50 min at 50°C. The apparent molecular weights of the purified liver and brain enzymes were 154 000 and 152 000, respectively. Hexosamines activated β-*N*-acetyl-hexosaminidase S whereas the isoenzyme A and B were inhibited. The glycoprotein nature of β-*N*-acetyl-hexosaminidase S was suggested by its affinity towards Concanavalin A-Sepharose.

Introduction

β-*N*-Acetylhexosaminidase (2-acetamido-2-deoxy-β-hexoside acetamido-deoxyhexohydrolase, EC 3.2.1.52) of human tissues exists in two major molecular forms A and B [1]. Minor isoenzymes such as I_1, I_2 [2] and P [3] have also been described. Forms C and S are both detected in tissues, body fluids and fibroblasts of patients with variant O G_{M2} gangliosidosis (Sandhoff's disease) [4—7]. In this disease, both A and B isoenzymes are deficient and forms C and S represent the 5—10% residual activity.

Isoenzyme C was first reported in human brain by Hoogwinkel et al. [8] and subsequently in various human tissues [9]; this form differs from A, B and S isoenzymes by numerous properties. The type C is localized in the cytosol rather than the lysosomal fraction, has neutral pH optimum, larger molecular size, very low β-*N*-acetyl-galactosaminidase activity and does not react with anti-

isoenzyme A or B serum [10]. These properties suggest a different genetic origin for this isoenzyme. Recently, Swallow et al. [6] reported that form C could be quantitated in tissues since, unlike isoenzymes A, B and S, this isoenzyme is not adsorbed on Concanavalin A-Sepharose.

Isoenzymes A and B have been purified to homogeneity from various human tissues [11—16] and several of their properties have been determined. A model of their subunit structure has been proposed [15,17] which postulates two subunit types α and β, coded by two different genes, and associated in the following tetrameric forms; B, β_4; A, $\alpha_2\beta_2$ and S, α_4. However, the confirmation of the subunit structure as well as the kinetic and physical properties of form S must await a detailed study of a purified preparation. This paper presents a method for the purification of form S from the liver and brain of a patient who died from Sandhoff's disease. The purified preparation was essentially free of form C contamination and some properties of the purified enzyme are reported.

Materials and Methods

Liver and brain from a 4-year old girl who died from type O G_{M2} gangliosidosis were obtained at autopsy (about 2 h after death) and stored at —60°C for 2 years (liver) or 4 years (brain). Some clinical and biochemical findings on this patient have been reported [18].

The 4-methylumbelliferyl-glycosides of β-2-acetamido-2-deoxy-D-glucopyranoside and β-2-acetamido-2-deoxy-D-galactopyranoside were purchased from Koch-Light Laboratories Ltd (Colnbrook, England). Glucosamine and galactosamine hydrochlorides, *N*-acetylglucosamine and *N*-acetylgactosamine were obtained from Calbiochem (La Jolla, Ca.). Fresh solutions were prepared each week and kept at —20°C. Sephadex G-25, DEAE-Sephadex A-25, Sulfopropyl-Sephadex C-25, Sepharose 6B, Concanavalin A-Sepharose, bovine serum albumin and ovalbumin were purchased from Pharmacia Fine Chemicals (Montréal). Rabbit muscle aldolase (grade I), Coomassie Blue R 250 and methyl-α-D-glucopyranoside were obtained from Sigma Chemicals Co. (St. Louis, Mo.).

Determinations of isoenzymes C and S in liver and brain

The hexosaminidase C and S content of tissue extracts was determined as described by Swallow et al. [6] using Concanavalin A-Sepharose chromatography. A small (1 × 5 cm) column was kept at 25°C and eluted with 0.01 M acetate buffer (pH 6.0), 0.1 M NaCl, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, 0.001 M $CaCl_2$. Type C activity was not retained on the column but type S could be eluted with the same buffer containing 10% methyl-α-D-glucopyranoside.

β-N-Acetyl-hexosaminidase assay

Hexosaminidase activity was determined by the method of Okada and O'Brien [19] with either 4-methylymgelliferyl-β-glucosaminide or -β-galactosaminide as substrate. Unless specifically stated, the glucosaminide derivative was used. Assay was performed at pH 4.4 instead of pH 5.0 (pH optimum of type S) to diminish the contribution of type C to substrate hydrolysis in crude preparations. Type C activity was reported to be maximum at pH 7.0 but was still about 40% of maximum at pH 5.0 and about 20% of maximum at pH 4.4 [10].

One unit of β-*N*-acetyl-hexosaminidase activity is the amount of enzyme that liberates 1 nmol 4-methylumbelliferone per min.

Protein determination

Protein concentrations were determined by the method of Böhlen et al. [20] using bovine serum albumin as standard. Small peptides or free amino acids in crude preparations were eliminated by dialysis against deionized water. All samples were treated with 0.1 M NaOH before assay.

Polyacrylamide gel electrophoresis was performed at 4°C according to the method of Davis [21] using a 5% separating gel at pH 8.9. After electrophoresis for 1 h, protein were stained with Coomassie Blue R-250 in 7% acetic acid.

To detect enzyme activity, unstained gels were cut in 4 mm pieces and enzyme activity was determined on each piece after homogenization in 0.04 M citrate buffer (pH 4.4).

Molecular weight estimation

The molecular weight of purified type S was determined by Sepharose 6B chromatography on 1.6 × 88 cm column equilibrated with 0.1 M sodium phosphate buffer (pH 6.0) [22]. The column was calibrated with the following standard proteins: aldolase, 158 000; bovine serum albumin, 67 000 and ovalbumin, 45 000.

Purification of liver isoenzyme S

All purification steps were carried out at 0—4°C.

Steps 1 and 2: 200 g liver was thawed, washed several times with cold 0.15 M NaCl and homogenized in a precooled Waring Blendor for 3 min in 800 ml deionized water. The homogenate was adjusted to pH 5 with 1 M citrate buffer before centrifugation at 8000 × *g* for 10 min.

Step 3: The precipitate at 40—70% saturation of $(NH_4)_2SO_4$ was collected and dissolved in a minimum volume of 0.01 M sodium phosphate buffer (pH 5.0), 0.05 M NaCl. Insoluble material was removed by centrifugation and the protein was desalted on Sephadex G-25.

Step 4: The fractions containing the β-*N*-acetyl-hexosaminidase activity were pooled and applied (50 ml) on a DEAE-Sephadex A-25 column (2.5 × 35 cm) equilibrated with 0.01 M glycylglycine buffer (pH 8), 0.05 M NaCl (flow rate, 25 ml/h). A linear gradient, 0.05—0.25 M NaCl, was used and fractions containing the β-*N*-acetyl-hexosaminidase activity peak (eluted at about 0.190 M NaCl) were pooled and dialysed by ultrafiltration (Amicon Corp., PM-10 membrane).

Step 5: This preparation was rechromatographed on DEAE-Sephadex A-25 as before (0.9 × 16 cm column, flow rate, 6 ml/h). Active fractions (eluted with 0.19 M NaCl) were pooled and concentrated by ultrafiltration.

Step 6: The solution was dialysed in the ultrafiltration cell against 0.01 M citrate buffer (pH 4.4), 0.05 M NaCl and applied on a SP-Sephadex C-25 column (0.9 × 18 cm) previously equilibrated with the same buffer. The breakthrough peak contained all β-*N*-acetyl-hexosaminidase activity applied to the column.

Step 7: After concentration by ultrafiltration, the preparation was applied

on a Sepharose 6B column (1.6 × 82 cm) equilibrated with phosphate/NaCl buffer (pH 5). The fractions containing the enzyme activity were pooled and concentrated by ultrafiltration to a protein concentration of 0.18 mg/ml. This preparation was stable at 4°C for at least 3 months in 0.2 mg/ml bovine serum albumin, 0.04 M citrate buffer (pH 4.4).

Purification of brain isoenzyme S

Form S was purified from the brain of our patient (356.1 g) using the procedure described for liver type S except that the SP-Sephadex chromatography step was omitted and that brain type S was further purified by a second chromatography on Sepharose 6B in 0.1 M sodium phosphate buffer (pH 6.0). Final purification was obtained by preparative electrophoresis on polyacrylamide gels according to Davis [20]. The protein band corresponding to type S activity was cut off and extracted thrice with 1.5 ml 0.04 M citrate buffer (pH 4.4). The extract was concentrated to 0.9 ml by dialysis against Aquacid (Calbiochem) followed by dialysis for 19 h at 4°C against 1 liter 0.1 M phosphate buffer, pH 6.0 (final protein concentration 0.19 mg/ml).

Results

A summary of the purification procedure of liver and brain β-*N*-acetyl-hexosaminidase S is presented in Table I. The estimation of the purification and yield

TABLE I

PURIFICATION OF *N*-ACETYL-β-HEXOSAMINIDASE S

	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
A. From liver					
1- Homogenate	965	24 024	1 250	0.052	100
2- Extract	600	3 490	964	0.27	77.1
3- Ammonium sulfate	52	11 690	674	0.40	53.9
4- DEAE-Sephadex	30	680	450	0.66	36.0
5- DEAE-Sephadex	10	150	335	2.21	26.8
6- SP-Sephadex	6.2	70	260	3.70	20.8
7- Sepharose 6B, pH 5	0.7	0.13	56	447	4.5 (7.0)*
B. From brain					
1- Homogenate	1 410	20 370	2 656	0.13	100
2- Extract	1 000	4 070	1 780	0.31	67.0
3- Ammonium sulfate	220	919	528	0.36	19.9
4- DEAE-Sephadex	18	111	51	0.46	1.9
5- DEAE-Sephadex	2	22.4	32	0.57	1.2
6- Sepharose 6B, pH 5	1.4	n.d.	18.2	n.d.	0.69
7- Sepharose 6B, pH 6	1.3	0.31	8.8	24.9	0.33
8- Polyacrylamide gel electrophoresis	0.9	0.17	2.1	123	0.08 (0.44)

* Values in parentheses are calculated yields assuming that 64% and 18% of total liver and brain β-*N*-acetyl-hexosaminidase activity are respectively due to type S.
n.d., not determined.

of type S activity was difficult since type S accounted for 64% of total acetyl hexosaminidase activity in the liver and only 18% of that in the brain as determined by Concanavalin A-Sepharose chromatography. The activity adsorbed on the column was due to Type S alone and could be eluted with 10% methyl-α-D-glucopyranoside. Taking these values into account, overall yield was 7.0% and purification about 13 500-fold for liver type S and 0.44% and 5240-fold for brain type S. The affinity of the S form for Concanavalin A-Sepharose suggests that this isoenzyme is a glycoprotein.

Polyacrylamide gel electrophoresis of liver type S after step 7 of the purification procedure revealed one major protein band corresponding to acetyl hexosaminidase activity and two minor contaminants. The S form from brain revealed three protein bands in addition to a major band corresponding to acetyl hexosaminidase activity. This major band was cut out from the gel and extracted and this preparation was used for further characterization of brain type S.

Purified brain acetyl hexosaminidase S showed maximum activity at pH 5.0 when either the glucosaminidase or the galactosaminidase derivative was used as substrate (Fig. 1). Similar results were obtained with the liver enzyme (not shown). Michaelis-Menten constants, K_m, obtained by plotting reciprocals of initial velocity of enzymatic reaction versus substrate concentration, were 0.8—0.9 mM with the glucosaminide and 0.3—0.4 mM with the galactosaminide for S forms from both liver and brain. Maximum velocity of the enzymatic reaction (V) for liver type S was 828 and 132 units/mg of protein with the glucosaminide and galactosaminide, respectively, and 227 and 34.8 units/mg of protein for brain type S.

The effect of heat treatment at 50°C on purified brain type S and on the enzyme activity in crude brain extract is shown in Fig. 2. About 70% of S type activity was lost after 50 min at 50°C but acetyl hexosaminidase activity was more labile in the crude brain extract than in the purified preparation. The

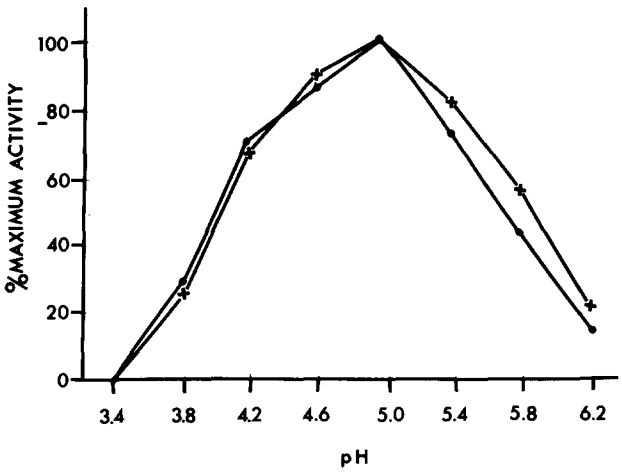
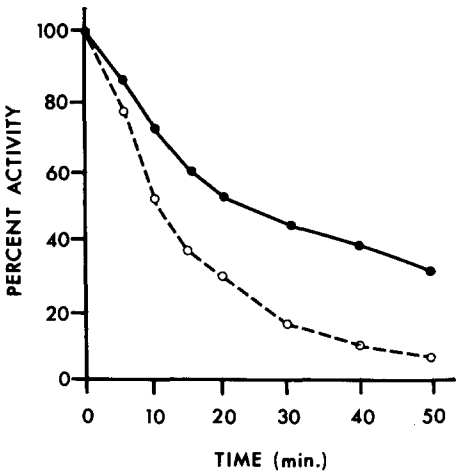


Fig. 1. pH-activity curve of purified brain β-*N*-acetyl-hexosaminidase S in 0.04 M citrate buffer. β-*N*-Acetyl-hexosaminidase (•———•); β-*N*-acetyl-galactosaminidase (X———X).



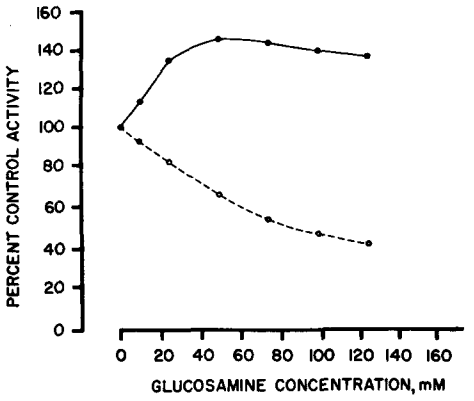


Fig. 2. Effect of preincubation time at 50°C on enzyme activity. The enzyme was preincubated in 0.04 M citrate buffer (pH 4.4), for different intervals of time, without substrate, and then assayed under standard assay conditions. Purified brain β-*N*-acetyl-hexosaminidase S (●———●); crude brain extract (○- - - - -○).

Fig. 3. Effect of varying glucosamine concentrations on purified liver β-*N*-acetyl-hexosaminidase S (●———●) and A (○- - - - -○) activities. The experiment was performed as described in the legend of Table II.

brain extract probably contains destabilizing agents which could be removed during the purification procedure. This effect could also be due to the C form which is thermolabile [10].

The effect of various substances on liver and brain type S is shown in Table

TABLE II

EFFECTS OF VARIOUS SUBSTANCES ON THE ACTIVITIES OF ISOENZYMES

β-*N*-Acetyl-hexosaminidase (0.04—0.05 unit) was incubated 10 min at 37°C in 0.04 M citrate buffer, pH 4.4, in presence of the compounds indicated in the table. After the incubation, enzyme activities were determined by addition of the substrate and further incubation for 20 min. The liver A and B form preparations were purified up to step 5 of the purification procedure (specific activity, type A: 185 units/mg protein; H type B: 13.5 units/mg protein).

	Percent control activity			
	Liver (S form)	Brain (S form)	Liver (A form)	Liver (B form)
N-Acetylglucosamine, 30 mM	40.7	19.9	19.9—24.6	20.9—30.5
N-Acetylgalactosamine, 30 mM	12.1	13.6	3.2—8.6	2.9—9.9
Glucosamine, 30 mM	135.0	110.7	72.2—76.3	65.7—73.3
Galactosamine, 30 mM	135.0	114.3	84.3—89.3	79.6—87.8
p-Chloromercuriphenyl-sulfonic acid, 1 mM	38.3	14.2	1.9—2.2	2.6—9.9
Acetate, 30 mM	39.8	71.4	33.3—39.4	30.6—38.2

II. *N*-Acetylhexosamines, *p*-chloromercuriphenylsulfonic acid and acetate were inhibitory for liver and brain type S and also for partially purified isoenzymes A and B from normal liver [11]. Unexpectedly however, both hexosamines activated the S form whereas isoenzymes A and B were inhibited. This effect was further studied using varying concentrations of glucosamine on liver isoenzymes S and A (Fig. 3). Even at the highest glucosamine concentration used (125 mM), liver type S activity was still 137% of control whereas type A was inhibited to 43% of control. The effect of glucosamine on Hex S is due to enhanced V (1158 units/mg protein) whereas the K_m value (1.1 mM) was not notably affected in the presence of 50 mM glucosamine. Isoenzyme S activation by hexosamines was also observed in crude liver extracts from this patient and from a Sandhoff's affected foetus (19 weeks of gestation, sister of the patient). That type S is not just stabilized but rather activated by hexosamines was ruled out by the observation that type S activity is stable for at least 30 min, under our assay conditions, in the presence or absence of 50 mM glucosamine.

The apparent molecular weight of liver and brain type S determined by gel chromatography on Sepharose 6B was 154 000 and 152 000, respectively.

Discussion

This paper describes a purification procedure for liver and brain type S to a high degree of purity. The kinetic and physical properties of purified type S were similar to those previously reported by other authors [4,5,23]. The S form preparations appeared essentially free of type C contamination. The purified S form showed maximum activity at pH 5.0 and the enzyme activity diminishes at higher pH of the incubation medium (Fig. 1) whereas type C activity was maximum around pH 7.0 [10]. The ratio of *N*-acetylglucosaminidase to *N*-acetylgalactosaminidase activities in purified type S was 4.7—4.8 whereas in the C form the ratio was 22—25 [10]. Finally, the apparent molecular weight of Form S determined by gel filtration (152 000—154 000) was lower than that reported for type C, about 190 000 [10]. Poenaru and Dreyfus [10] reported that type C is precipitated by 0—30% saturation of $(NH_4)_2SO_4$ whereas we retained acetyl hexosaminidase activity which precipitated at 40—70% saturation of $(NH_4)_2SO_4$. In addition, type C was not recovered from DEAE-ion exchanger columns [10].

A model of the subunit structure of acetyl hexosaminidase isoenzymes has been proposed in which isoenzyme B is β_4, A, $\alpha_2\beta_2$ and S, α_4 [15,17]. That type S subunit structure is a homopolymer of α chains was implicated by the observation that the A form could be converted into the B and S forms through dissociation and reassociation of its subunits [24]. The subunit structure of the purified S form cannot be studied because of the small amount of purified enzyme available. However, the finding that type S is activated by hexosamines (Table II, Fig. 3), without increase in the K_m value, suggests that the α subunit possesses a binding site for hexosamines distinct from the active site, and that binding enhances the rate of substrate hydrolysis. This effect could be caused by direct modification of the enzyme conformation at the active site or through reassociation or dissociation of the subunits.

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