Hydrolysis of Tay-Sachs Ganglioside by $\beta$-Hexosaminidase A of Human Liver and Urine*

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SUMMARY
A crude $\beta$-hexosaminidase fraction prepared by (NH$_4$)$_2$SO$_4$ fractionation of human liver extract or urine was found to convert Tay-Sachs ganglioside, GalNAc$\beta$1$\rightarrow$4(NANA$\alpha$2$\rightarrow$3)Gal$\beta$1$\rightarrow$4Glc$\rightarrow$ceramide (G$_{M3}$) into NANA$\alpha$2$\rightarrow$3Gal$\beta$1$\rightarrow$4Glc$\rightarrow$ceramide (G$_{M2}$). After separation of hexosaminidase A and B by DEAE-cellulose chromatography, only freshly prepared $\beta$-hexosaminidase A hydrolyzed G$_{M3}$ although both forms were still active toward $p$-nitrophenyl $\beta$-D-N-acetylglucosaminide. A heat-stable, nondialyzable preparation obtained from the crude $\beta$-hexosaminidase fraction of human liver was found to stimulate the hydrolysis of G$_{M2}$ by $\beta$-hexosaminidase A but not B isolated from both sources. Upon aging, $\beta$-hexosaminidase A would only hydrolyze G$_{M2}$ in the presence of the heat-stable preparation. Extensive purification as well as aging tended to reduce the capacity of $\beta$-hexosaminidase A to hydrolyze G$_{M2}$ even in the presence of the heat-stable preparation. Our results explain why $\beta$-hexosaminidase A has been previously reported by other investigators to hydrolyze G$_{M2}$ only with great difficulty. Our results also relate the inordinate storage of G$_{M2}$ to the absence of $\beta$-hexosaminidase A in the classical form of Tay-Sachs disease.

Human tissues contain two $\beta$-hexosaminidase isozymes, $\beta$-hexosaminidase A and B (1) Okada and O'Brien (2), Sandhoff (3), and Hultberg (4) demonstrated that $\beta$-hexosaminidase A was deficient in Tay-Sachs disease, a result which was based on the measurement of $\beta$-hexosaminidase activity of various tissues. Although it has been reported that G$_{M2}$ could be hydrolyzed by highly purified $\beta$-hexosaminidase isolated from various tissues (5-7), in all cases, the rate of hydrolysis was extremely slow and required the use of radioactive G$_{M2}$ if hydrolysis was to be detected at all. Weuger et al. (8) recently examined the substrate specificity of $\beta$-hexosaminidase A and B isolated from human liver and concluded that neither enzyme hydrolyzed the terminal N-acetylgalactosaminylic unit from G$_{M2}$. In order to elucidate the relationship between the inordinate storage of G$_{M2}$ and the absence of $\beta$-hexosaminidase A in Tay-Sachs disease, we have made an extensive examination of the action of the $\beta$-hexosaminidases isolated from human liver and urine upon G$_{M2}$.

EXPERIMENTAL PROCEDURE

*p-Nitrophenyl-$\beta$-D-N-acetylglucosaminide was purchased from Sigma Chemical Company. G$_{M3}$ was isolated from normal human brain (9) and G$_{M2}$ from human plasma (10). Radioactive G$_{M2}$ (2300 cpm per nmole), $^3$H-labeled in the terminal N-acetylgalactosamine, was a gift of Professor Lars Svennerholm of the University of Göteborg in Sweden. The asialo derivative of G$_{M2}$ was prepared by hydrolyzing G$_{M2}$ with 1 M HCOOH for 1 hour at 100° as previously described (11). Globo-side was prepared from human red cell stroma (12). Analytical thin layer chromatography of gangliosides and neutral sphingoglycolipids was performed on plates coated with a layer (0.25 mm) of Silica Gel G and developed with chloroform-methanol-water (60:32:7 or 65:25:4). Gangliosides and neutral sphingoglycolipids were visualized by spraying the plates with anisaldehyde (13) or resorcinol reagent (14). For convenience, $p$-nitrophenyl-$\beta$-D-N-acetylglucosaminide was used routinely to follow $\beta$-hexosaminidase activity during enzyme isolation. The procedure for using $p$-nitrophenyl-$\beta$-D-N-acetylglucosaminide as substrate has been described elsewhere (15). One unit of enzyme was defined as the amount of enzyme which hydrolyzes 1 µmole of $p$-nitrophenyl-$\beta$-D-N-acetylglucosaminide per min at 37°. The specific activity of the enzyme was expressed as units per mg of protein. Protein was determined by the method of Lowry et al. (16) with crystalline bovine serum albumin as the standard. The preparations obtained at different purification stages were characterized by thin layer chromatography, using asialo-GM$_2$, galactoside, GM$_2$, and GM$_3$ as standard substrates.
steps were then separately tested for their ability to hydrolyze \( \text{GM}_2 \) and other sphingolipids. The procedures for hydrolyzing sialic acid from \( \text{GM}_2 \) with cholera neuraminidase and galactose from lactosylceramide with jack bean \( \beta \)-galactosidase have been previously described (10, 11).

**Isolation of \( \beta \)-Hexosaminidases from Human Liver—**Enzyme isolation was carried out at 0-5°. Human liver (400 g) obtained post mortem was diced, homogenized with 2400 ml of 0.1% (v/v) Triton X-100, in a Waring blender at 12,500 rpm for 2 min and centrifuged at 8,000 \( \times \) g to obtain a clear brown crude extract. Solid citric acid was added to adjust the pH of the extract to 4.3 while stirring. After standing overnight, the precipitate was removed by centrifugation and discarded. The pH of the supernatant was adjusted with solid tris(hydroxymethyl)aminomethane to pH 7.0 and was then brought to 30% saturation with solid (NH\(_4\))\(_2\)SO\(_4\). After settling overnight, the precipitate was centrifuged to remove the precipitate, and the supernatant was brought to 50% saturation with solid (NH\(_4\))\(_2\)SO\(_4\).

The precipitated protein was harvested the next day by centrifugation and resuspended in 35 ml of 0.01 M sodium phosphate buffer, pH 6.0. This preparation was designated as the crude \( \beta \)-hexosaminidase. No neuraminidase activity capable of releasing sialic acid from \( \text{GM}_3 \), \( \text{GM}_4 \), or \( \text{GM}_5 \) was detectable in this preparation.

Partially purified \( \beta \)-hexosaminidase A and B were prepared from the crude \( \beta \)-hexosaminidase by DEAE-cellulose chromatography according to the procedure described by Robinson and Stirling (1). Partially purified \( \beta \)-hexosaminidase A was further purified by CM-cellulose chromatography. After dialysis against 0.05 M sodium citrate buffer, pH 4.4, the enzyme was applied to a CM-cellulose column equilibrated with the same buffer. After washing the column with the same buffer, the enzyme was eluted with a linear salt gradient from 0 to 0.4 M NaCl in the same buffer.

The fractions containing \( \beta \)-hexosaminidase activity were pooled, precipitated by reverse dialysis against (NH\(_4\))\(_2\)SO\(_4\) and designated as highly purified liver \( \beta \)-hexosaminidase A. This procedure is summarized in Table I.

**Heat-stable Nondialyzable Preparation from Crude \( \beta \)-Hexosaminidase—**The crude \( \beta \)-hexosaminidase isolated from human liver was heated at 100° for 5 min and centrifuged at 17,000 \( \times \) g for 20 min to obtain a clear supernatant. This supernatant, after exhaustive dialysis against distilled water and lyophilization, was designated as the heat-stable preparation. This preparation alone does not have any activity toward \( \text{GM}_3 \) or \( \text{p-Nitrophenyl-}\beta-N \)-acetylglucosaminidase.

**Isolation of \( \beta \)-Hexosaminidases from Human Urine—**Fresh urine from healthy male subjects was pooled and made 60% saturated with (NH\(_4\))\(_2\)SO\(_4\). After standing in the cold (4°) overnight, the clear supernatant was removed by decantation and the thick slurry at the bottom of the container was centrifuged to obtain a peaked dark brown precipitate. The yield of this precipitate was about 10 to 15 g/10 liters of urine. The precipitate obtained from 100 liters of urine was pooled and extracted twice with 500 ml of 0.05 M sodium phosphate buffer, pH 7.0. The \( \beta \)-hexosaminidase in the extracts was then precipitated between 35 to 65% saturation with (NH\(_4\))\(_2\)SO\(_4\). The precipitate was dissolved in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0, and was designated as crude urine extract. The crude urine extract (20 ml) was applied to a Sephadex G-200 column (5 x 90 cm) previously equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The column was eluted with the same buffer. Those fractions containing \( \beta \)-hexosaminidase activity were pooled and designated as the crude urine \( \beta \)-hexosaminidase. Partially purified urine \( \beta \)-hexosaminidase A and B were also prepared from the crude \( \beta \)-hexosaminidase fraction by DEAE-cellulose chromatography according to Robinson and Stirling (1). \( \beta \)-Hexosaminidase A obtained at DEAE-chromatography was pooled and dialyzed against 0.05 M sodium acetate buffer, pH 4.4, and applied to a CM-cellulose column (2 x 30 cm) equilibrated previously with the same buffer.

After washing the column with the starting buffer and then with 0.05 M sodium acetate, pH 4.9, the \( \beta \)-hexosaminidase was eluted with 0.01 M citrate buffer, pH 6.2. This preparation was designated as highly purified urine \( \beta \)-hexosaminidase A. This procedure is summarized in Table I.

### Table I

**Purification of \( \beta \)-Hexosaminidases from 400 g of Human Liver and 100 liters of Human Urine**

| Steps | Total Units | Specific Activity (units/mg) |
|-------|-------------|-----------------------------|
| Liver |             |                             |
| Crude extract | 1900 | 0.09 |
| Crude \( \beta \)-Hexosaminidase | 945 | 0.43 |
| DEAE-cellulose column | 447 | 1.1 |
| \( \beta \)-Hexosaminidase A | 124 | 0.24 |
| CM-cellulose column (\( \beta \)-Hexosaminidase A) | 310 | 11.2 |
| Urine |             |                             |
| Crude extract | 170 | 0.19 |
| Sephadex G-200 filtration | 130 | 0.60 |
| DEAE-cellulose column | 56 | 3.2 |
| \( \beta \)-Hexosaminidase A | 25 | 1.1 |
| \( \beta \)-Hexosaminidase B | 38 | 10.2 |

Enzymatic Hydrolysis of \( \text{GM}_3 \), Asialo \( \text{GM}_3 \), and Globoside—Enzyme activity was assayed in the presence or absence of 2 mg (dried weight) of the heat-stable preparation. When \( \text{GM}_3 \) was used as substrate, 20 nmoles in 100 \( \mu \)l of chloroform-methanol (1:1) were evaporated to dryness under a stream of nitrogen. Then, 0.7 ml of 0.05 M sodium citrate buffer, pH 4.0, was added and the mixture was sonicated in a Cole-Parmer model 8845-3 ultrasonic bath for 3 min. The reaction was started by adding 0.1 ml of enzyme solution containing 1 to 10 units of enzyme and incubated at 37° for 2 to 16 hours. The reaction was stopped by the addition of 4 volumes of chloroform-methanol (1:1). After thorough mixing, the chloroform phase and the aqueous phase were separated by centrifugation. Under these conditions, more than 90% of the \( \text{GM}_3 \) and \( \text{GM}_4 \) was recovered in the lower phase. The lower phase was carefully removed, evaporated to dryness under a stream of nitrogen and analyzed by thin layer chromatography. When asialo \( \text{GM}_3 \) or globoside was used as substrate, the procedure was essentially the same as above except that the buffer contained 100 \( \mu \)g of sodium taurocholate per 100 \( \mu \)l and the reaction was terminated by the addition of 4 volumes of chloroform-methanol (2:1) as previously described (11). For the quantitative determination of the hydrolysis of \( \text{GM}_3 \), 20 nmoles of [\( \text{GalNAc-}^3\text{H}\text{GM}_3 \) (46,000 cpm)] was incubated with either 1.2 units of \( \beta \)-hexosaminidase A or 1.5 units of \( \beta \)-hexosaminidase B for 8 hours under the same conditions described for the hydrolysis of nonradioactive \( \text{GM}_3 \). After incubation, the unreacted ganglioside was co-precipitated with human serum albumin by trichloroacetic acid according to the
procedure described by Tallman and Brady (17). Radioactive N-acetylgalactosamine in the supernatant was subsequently determined by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Electrophoretic examination (18) revealed that $\beta$-hexosaminidase A and B of liver and urine were completely separated at the DEAE-chromatography step. With $p$-nitrophenyl-$\beta$-d-$N$-acetylglucosaminide as substrate, the pH optimum was between 4.0 and 4.4 for both $\beta$-glucosaminidase A and B of human liver and urine. For hydrolysis of G$_{M2}$ by the crude $\beta$-hexosaminidase of human liver and urine, the pH optimum was between 3.8 and 4.0.

All $\beta$-hexosaminidase preparations from liver and urine obtained at different purification steps hydrolyzed $p$-nitrophenyl-$\beta$-d-$N$-acetylglucosaminide, globoside, and the asialo derivative of G$_{M2}$. However, only the crude $\beta$-hexosaminidase obtained at the (NH$_4$)$_2$SO$_4$ fractionation step efficiently converted G$_{M2}$ into G$_{M3}$. This conversion of G$_{M2}$ to G$_{M3}$ by the crude $\beta$-hexosaminidase of human liver is shown in Fig. 1. Hydrolysis as a function of G$_{M2}$ concentration was also investigated. Visual examination of the plate clearly indicated that the amount of product was a function of substrate concentration and that only a typical enzyme reaction. Hydrolysis was roughly linear with time at a given G$_{M2}$ concentration. At the substrate concentration of 11 $\mu$M, about 30% of the G$_{M2}$ was converted to G$_{M3}$ by 5 units of crude liver $\beta$-hexosaminidase in 16 hours under the standard assay conditions which may not be the optimal condition for hydrolyzing G$_{M2}$ by liver and urine hexosaminidase.

In Fig. 2A, the slow hydrolysis of G$_{M2}$ to G$_{M3}$ by partially purified liver $\beta$-hexosaminidase A is illustrated. The increased hydrolysis of G$_{M2}$ by $\beta$-hexosaminidase A in the presence of the heat-stable preparation is also shown. Similar results were also obtained with the corresponding urine $\beta$-hexosaminidase A preparation (Fig. 2B). While freshly prepared $\beta$-hexosaminidase A of urine or liver obtained by DEAE-cellulose chromatography still retained some ability to cleave G$_{M2}$, the corresponding $\beta$-hexosaminidase B was not able to hydrolyze G$_{M2}$ even in the presence of the heat-stable preparation. It is of interest to notice that highly active crystalline jack bean $\beta$-hexosaminidase (15) could not hydrolyze G$_{M2}$ under any circumstances. Sodium taurocholate could not replace the heat-stable preparation in stimulating the hydrolysis of G$_{M2}$ by $\beta$-hexosaminidase.

Liver $\beta$-hexosaminidase A, after further purification by CM-cellulose chromatography, was completely inactive toward G$_{M2}$.

![Fig. 1. Conversion of G$_{M2}$ to G$_{M3}$ by the crude $\beta$-hexosaminidase of human liver. The enzyme (5 units) was incubated with 20 nmoles of G$_{M2}$ in 0.8 ml of 0.05 M sodium citrate buffer, pH 4.0, at 37°C for 15 hours. Detailed incubation conditions are described in the text. E, crude liver $\beta$-hexosaminidase.](http://www.jbc.org/)

![Fig. 2. A, hydrolysis of G$_{M2}$ by crystalline jack bean $\beta$-hexosaminidase and partially purified $\beta$-hexosaminidase A and B of human liver in the presence and absence of the heat-stable preparation. Jack bean $\beta$-hexosaminidase (36 units), $\beta$-hexosaminidase A (1.2 units), and $\beta$-hexosaminidase B (1.3 units) were separately incubated with G$_{M2}$ (23 $\mu$M) in the presence or absence of 2 mg (dried weight) of the heat-stable preparation at 37°C for 16 hours. The plate was sprayed with anisaldehyde. JB Hex, jack bean $\beta$-hexosaminidase; Hex-A or B, liver $\beta$-hexosaminidase A or B; Sup, the heat-stable preparation; STD, standard. B, hydrolysis of G$_{M2}$ by partially purified $\beta$-hexosaminidase A and B of human urine. $\beta$-Hexosaminidase A (1.2 unit) and $\beta$-hexosaminidase B (1.5 unit) were separately incubated with G$_{M2}$ (23 $\mu$M) in the presence or absence of 2 mg (dried weight) of the heat-stable preparation at 37°C for 16 hours. For the conditions for incubating G$_{M2}$ with crude $\beta$-hexosaminidase, see text. The plate was sprayed with resorcinol. Liver enzyme, crude liver $\beta$-hexosaminidase.](http://www.jbc.org/)
The ability of this preparation to hydrolyze GM₃, however, could be restored by adding the heat-stable preparation when the highly purified enzyme was fresh. After storage at 4°C for 5 days, this preparation could no longer be stimulated by the heat-stable factor to hydrolyze GM₃, although it still retained the ability toward p-nitrophenyl-β-D- Glucosaminidase. Similar results were also obtained for the corresponding β-hexosaminidase A preparation isolated from urine. Finally, the recombination of β-hexosaminidase A and B without the heat-stable preparation did not restore the capacity to hydrolyze GM₃.

By using radioactive GM₃, the partially purified β-hexosaminidase A isolated from human liver hydrolyzed 0.3 nmoles of GM₃ per hour per unit of enzyme in the presence of the heat-stable preparation. In the absence of the heat-stable preparation, it only hydrolyzed 0.06 nmoles of GM₃ per hour per unit of enzyme. The freshly prepared β-hexosaminidase A purified by CM-cellulose chromatography hydrolyzed 0.06 nmoles of GM₃ per hour per unit of enzyme in the presence of the heat-stable preparation. In the absence of the heat-stable preparation, no significant hydrolysis was observed. Under the same condition, β-hexosaminidase B did not hydrolyze GM₃ in the presence or absence of the heat-stable preparation. Sandhoff (6) reported that a highly purified β-hexosaminidase A (enriched 3000-fold) of human liver hydrolyzed GM₃ at a rate of 0.04 nmoles per hour per unit of enzyme in the presence of sodium taurocholate. The reason for the low rate of hydrolysis could be due to the removal of the heat-stable factor during the course of purification. Sandhoff also found that GM₃ was not hydrolyzed by liver β-hexosaminidase B. Wenger et al. (8) recently reported that they could not detect the hydrolysis of GM₃ by either β-hexosaminidase A or B isolated from human liver. The reason for their negative result could be due to the fact that their enzymes were isolated from the frozen tissues stored at −20°C for 1 to 3 years. We found that freezing and aging of the tissue greatly reduced the ability of β-hexosaminidase A to hydrolyze GM₃.

As can be seen from Figs. 1 and 2, the mobility of GM₃ produced from brain GM₃ was slightly faster than the standard GM₃ isolated from human plasma. This is clearly due to the differences in their fatty acid composition. While stearic acid is the major fatty acid of sphingolipids isolated from brain, nervonic and palmitic acids are the major fatty acids found in the sphingolipids of plasma (10). The identity of the product as GM₃ was confirmed by the following: (a) hydrolysis of the chloroform-methanol extract of the reacted mixture with 1 M HCOOH for 1 hour produced asialo GM₃ from unreacted GM₃ and lactosylceramide from GM₃; (b) the standard GM₃ isolated from human plasma (10) and the GM₃ produced from GM₃ were both converted to lactosylceramide by neuraminidase from Vibrio cholera; and (c) the lactosylceramide produced by neuraminidase, in both cases, was further converted to glucosylceramide by jack bean β-galactosidase (11).

The fact that the heat-stable preparation activates β-hexosaminidase A to hydrolyze GM₃ suggests the presence of a “factor” in this preparation. This factor is apparently removed during the purification of the enzyme. Work on the isolation and characterization of this factor is in progress and will be published elsewhere. It is of interest to note that Ho et al. (19) have reported a low level of glucocerebrosidase activity (toward glucocerebroside) in a particular fraction of human spleen which is greatly stimulated by the addition of a soluble glycoprotein factor. They also observed a discrepancy between the ability of their particulate fraction to hydrolyze glucocerebroside and 4-methylumbelliferyl-β-glucoside. Similarly, it appears that full β-hexosaminidase activity towards GM₃ may require the interaction of two components, the purified β-hexosaminidase A enzyme and a heat-stable factor. It is evident that a glycosidase whose purification has been followed using synthetic substrates may not necessarily act on natural substrates. This fact cannot be overemphasized. Related to this is the fact that storage of purified β-hexosaminidase A in the cold has an adverse effect on its ability to hydrolyze GM₃ but not p-nitrophenyl-β-D-N-acetylglucosaminidase. Our results explain why previous reports by other investigators indicated hydrolysis of GM₃ only with great difficulty when using highly purified β-hexosaminidase A.

There are two possible catabolic pathways for GM₃.

I. GalNAcβ1 → 4Galα1 → 4Glc → Cer  β-hexosaminidase

II. GalNAcβ1 → 4Galα1 → 4Glc → Cer  α-neuraminidase

Our results suggest the existence of pathway I in human liver. The enzyme responsible for this pathway is β-hexosaminidase A. In addition, our results establish why the absence of β-hexosaminidase A causes the accumulation of GM₃ in various tissues of the classical Tay-Sachs patient.

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