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Diagnosis and Carrier Detection of Tay-Sachs Disease: Direct Determination of Hexosaminidase A Using 4-Methylumbelliferyl Derivatives of β-*N*-Acetylglucosamine-6-Sulfate and β-*N*-Acetylgalactosamine-6-Sulfate

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SUMMARY

4-Methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy derivatives of β-D-glucopyranoside and β-D-galactopyranoside were prepared by direct sulfation of the commonly used unsulfated derivatives. Both sulfated substrates were highly specific for hexosaminidase A, and in fractionated serum, cells, and tissue preparations, less than 2.5% of these activities were associated with hexosaminidase B and the intermediate isozyme fractions. Serum and leukocytes from patients with infantile Tay-Sachs disease, including a patient with thermolabile hexosaminidase B, had less than 2% of noncarrier activities. Carrier values were clearly separated from those of noncarriers, and no problems were encountered in utilizing sera from pregnant women. The % hexosaminidase A values as derived from the ratio between the activities toward the sulfated and unsulfated substrates in the same specimen were comparable to those obtained by the heat-inactivation method (except for subjects with thermolabile hexosaminidase B) and may be helpful in genotype determination in borderline cases.

INTRODUCTION

Tay-Sachs disease (TSD) or G_{M2} gangliosidosis type B (McKusick no. 27280) is an autosomal recessive inherited disorder of glycolipid catabolism caused by

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deficient activity of the lysosomal enzyme hexosaminidase A (HEX A), 2-acetamido-2-deoxy-β-D-gluco(galacto)side acetamidodeoxygluco(galacto)hydrolase, E.C.3.2.1.52 [1]. HEX A is a hetero-oligomer composed of two distinct subunits (α and β_2) that are coded by different loci on different chromosomes [2, 3]. The α subunit of the mature enzyme contains one polypeptide chain (M_r 54 kDa) and the β_2 subunit contains two nonidentical polypeptide chains of similar size β_a and β_b (M_r 28 and 27 kDa, respectively) [4, 5]. The deficiency of HEX A in TSD presumably results from mutation of the structural gene for the α-chain. The other major isozyme, HEX B, which possesses a higher isoelectric point, and the intermediate forms, HEX I_1, I_2, and P, are β-chain oligomers [6, 7]. They are not affected by the mutations in TSD, although their levels are proportionally increased due to the absence of α-chains.

All HEX isozymes are capable of cleaving synthetic chromogenic and fluorogenic β-*N*-acetylglucosaminides and β-*N*-acetylgalactosaminides. The commonly used differential test is indirect, based on the relative thermolability of HEX A as compared with HEX B, I_1, and I_2 [8, 9]. The use of the HEX A-specific G_{M2}-ganglioside substrate is very laborious and therefore not suitable for screening purposes. Kresse et al. [10] and Fuchs et al. [11] reported that *p*-nitrophenyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside (pNP-β-GlcNAc-6-S) can be specifically hydrolyzed by HEX A to yield free *p*-nitrophenol and *N*-acetylglucosamine-6-sulfate, and this direct and simple assay is suitable for diagnosis and carrier detection of TSD. Using this method, Li et al. [12] were able to differentiate between activator-deficient and HEX A-defective variants of G_{M2} gangliosidosis type AB. Inui and Wenger [13] and Bayleran et al. [14] recently reported that the corresponding 4-methylumbelliferyl fluorogenic substrate is useful for diagnosis and carrier detection of G_{M2} gangliosidoses resulting from mutations at the α locus. We report here that the 4-methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy derivatives of both β-D-glucopyranoside (MU-β-GlcNAc-6-S) and β-D-galactopyranoside (MU-β-GalNAc-6-S) are useful substrates for direct determination of HEX A. These assays are especially useful for detection of TSD patients and carriers with thermolabile HEX B and for TSD carrier detection in serum during pregnancy.

MATERIALS AND METHODS

Materials

DEAE-cellulose (Cellex-D), Ecteola-cellulose (Cellex-E), AG 50 WX8, and Bio-Gel P-2 were from Bio-Rad, Richmond, Calif. Pyridine and chlorosulfonic acid were from Mallinckrodt, Paris, Ken. 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MU-β-GlcNAc) and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside (MU-β-GalNAc) were from Sigma, St. Louis, Mo.

Enzyme Source

Sera and leukocytes were prepared as described [15]. Placenta and liver extracts were prepared by homogenizing minced tissue in 10 mM sodium phosphate buffer, pH 6.0 (4 ml/g of tissue). The homogenate was centrifuged at 12,500 *g* for 1 hr and the supernatant

collected. HEX isozymes (B, I_1, I_2, and A) were fractionated on a DEAE-cellulose column with a linear gradient of 0–0.5 M NaCl in 10 mM sodium phosphate buffer, pH 6.0, as described [15]. Protein concentration was measured by the dye-binding method of Bradford [16] using bovine γ-globulin as a standard (Bio-Rad).

Preparation of Sulfated Substrates

MU-β-GlcNAc-6-S and MU-β-GalNAc-6-S were synthesized according to the method of Lloyd [17]. A mixture of 65 μl (1 mmol) of chlorosulfonic acid and 365 μl of chloroform were added dropwise to an ice-cold stirring solution of 300 mg (0.8 mmol) MU-β-GlcNAc or MU-β-GalNAc in 10 ml of freshly distilled pyridine. The mixture was stirred at 0°C for 1 hr and at room temperature (23°C) for an additional 2 hrs. Two ml of H_2O were added to decompose the remaining chlorosulfonic acid, and the mixture was neutralized with 2 M NaOH. The mixture was dried in vacuo, redissolved in 5 ml of H_2O, and chromatographed on a DEAE-cellulose column (2.4 × 32 cm) with a wash of 250 ml of 25 mM pyridine/acetic acid, pH 5.0, and a linear gradient of 500/500 ml of 25–375 mM pyridine/acetic acid, pH 5.0. Fluorescence of 4-methylumbelliferyl compounds was detected by a Perkin-Elmer fluorescence spectrophotometer with excitation at 320 nm and emission at 375 nm. Aliquots of fluorescent fractions were hydrolyzed (4 N HCl, 4 hrs, 110°C), dried in vacuo, and analyzed for the content of 4-methylumbelliferone (excitation at 365 nm and emission at 450 nm), hexosamine [18], and sulfate [19]. Fractions containing equimolar quantities of the three components (eluted between 90 and 150 mM pyridine/acetic acid, pH 5.0) were pooled, dried in vacuo, redissolved in 5 ml of H_2O, and desalted on a Bio-Gel P-2 column (1.4 × 120 cm). The fluoresent fractions were freeze-dried, redissolved in 5 ml of H_2O, and the pyridinium salt of the sulfated compounds was converted to a sodium salt by passing through an AG 50 WX8, 200–400 mesh column (1.2 × 40 cm) followed by neutralization with 0.1 N NaOH. The fluorescent fractions were freeze-dried, and the yield was 148–170 mg (38.8%–44.6% of the theoretical yield). Repurification of the substrates prior to use was performed on an Ecteola-cellulose (formate) column (0.5 × 5 cm) using 0.2-ml aliquots. Unsulfated derivatives were washed with H_2O, and the sulfated substrate was eluted with 70 mM sodium formate.

Hexosaminidase Assays

HEX activity toward MU-β-GlcNAc-6-S and MU-β-GalNAc-6-S was determined with a final concentration of 2 mM substrate in 100 μl of 0.1 M sodium formate buffer, pH 4.3, and HEX activity toward MU-β-GlcNAc and MU-β-GalNAc with a final substrate concentration of 2 mM in 100 μl of citrate-phosphate buffer, pH 4.3 (0.1 M citric acid/0.2 M Na_2HPO_4). The reaction mixtures contained 10 μl of serum, 4–20 μg protein of leukocyte sonicate, or 1–5 μg protein of tissue extract. Reactions with sulfated substrates were incubated for 60–120 min at 37°C and with unsulfated substrates for 10–20 min at 37°C. The reactions were terminated by addition of 0.9 ml of 0.25 M glycine/Na_2CO_3 buffer, pH 10.2, and the fluorescence of the liberated 4-methylumbelliferone was measured at excitation wavelength of 365 nm and emission at 450 nm. Blanks were prepared by incubating separately enzyme source and substrate and mixing them together with the glycine/Na_2CO_3 buffer at the time of termination. Heat-inactivation was performed by incubation of samples in citrate-phosphate buffer, pH 4.3, for 2 hrs at 50°C.

RESULTS

Substrate Specificity

Hexosaminidase activities toward MU-β-GlcNAc-6-S and MU-β-GalNAc-6-S were linear with incubation time for at least 2 hrs and proportional to the

amount of added enzyme source up to 20 μl of serum and up to 50 μg protein of cell or tissue preparations. Optimum activity was determined at pH 4.0–4.5, and the apparent K_m values were below 0.5 mM. Both sulfated substrates were highly specific for HEX A, and as shown in table 1 for isozymes fractionated from placenta extract, the activities of HEX B, I_1, and I_2 amounted to less than 1% of the activity determined in the HEX A fraction. Taking into account the ratio of HEX A activities toward sulfated and unsulfated substrates (3.48 for MU-β-GlcNAc/MU-β-GlcNAc-6-S and 2.54 for MU-β-GalNAc/MU-β-GalNAc-6-S), HEX B, I_1, and I_2 exhibited about 0.5%, 2%, and 4% of the sulfated substrate degrading activity of HEX A, respectively. Similar results were also obtained for isozyme fractions from serum, leukocytes, and liver extract. More than 97.5% of the total activity toward either sulfated substrate was found in the HEX A fraction.

TSD Diagnosis

Serum and leukocytes from Jewish and non-Jewish patients with infantile TSD demonstrated less than 2% of the mean of the respective noncarrier activities toward the sulfated fluorogenic substrates (tables 2 and 3). Less than 2% of HEX A were also calculated as % of total HEX activity in these specimens, based on the ratio of activities toward sulfated and unsulfated substrates (table 4). Comparable, although somewhat higher, % HEX A values were calculated on the basis of heat-inactivation. HEX A levels in serum from an additional non-Jewish TSD patient with thermolabile HEX B were less than 2% of the mean noncarrier activity and less than 2% of total HEX activity when assayed directly with the sulfated and unsulfated substrates, but strictly based on the differential heat-inactivation method, one would assume that the patient has 24% HEX A (table 4).

TSD Carrier Detection

HEX A activity as determined with MU-β-GlcNAc-6-S and MU-β-GalNAc-6-S in sera and leukocytes from obligate TSD carriers were clearly separated from the respective values in noncarrier specimens (tables 2 and 3). Mean carrier activities were 55%–59% of the respective noncarrier means. The values of % HEX A from total HEX activity in the same specimen, as derived from sulfated/unsulfated activity ratios, were comparable to those obtained by the heat-inactivation method except for the case of TSD carrier with thermolabile HEX B who obviously demonstrated higher % of heat-labile HEX (which usually corresponds to HEX A) by the differential heat-inactivation method (table 4). The value obtained in this specimen by heat-inactivation was in the inconclusive range, whereas that obtained by sulfated/unsulfated activity ratio was clearly in the range of TSD carriers. Eleven noncarrier pregnant women who express what appeared to be carrier values for % serum HEX A by heat-inactivation had even lower % serum HEX A values by sulfated/unsulfated activity ratio, but the straight activity values determined in the serum with the sulfated substrates were clearly in the noncarrier range (table 4).

TABLE 1

HEX ACTIVITIES OF FRACTIONATED PLACENTA ISOZYMES TOWARD SULFATED AND UNSULFATED FLUOROGENIC SUBSTRATES

ISOZYME FRACTION	HEX ACTIVITY (nmol hr $^{-1}$)				
	MU-β-GlcNAc	(Heat-stable)*	MU-β-GalNAc	MU-β-GlcNAc-6-S	MU-β-GalNAc-6-S
HEX B . .	1,300	(1,243)	157	1.9	0.3
HEX I_1 .	105	(96)	12	0.4	0.1
HEX I_2 .	60	(44)	7	0.6	0.1
HEX A .	2,130	(51)	250	612	98.4

* MU-β-GlcNAc activity following 2-hr incubation at pH 4.3 and 50° C.

TABLE 2

SERUM HEX ACTIVITIES TOWARD SULFATED AND UNSULFATED FLUOROGENIC SUBSTRATES

GENETIC STATUS	HEX ACTIVITY (nmol hr $^{-1}$/ml $^{-1}$)			
	MU-β-GlcNAc	MU-β-GalNAc	MU-β-GlcNAc-6-S	MU-β-GalNAc-6-S
Noncarriers (no. = 33):				
Mean ± SD	701 ± 132	84.8 ± 15.9	130 ± 25	21.7 ± 4.7
Range	494–1,045	59.9–123	93–180	16.8–28.0
TSD-carriers (no. = 19):				
Mean ± SD	608 ± 67	73.0 ± 8.0	71.4 ± 7.0	12.8 ± 1.8
Range	509–743	62.0–90.2	58.8–83.5	9.8–16.4
TSD-patients (no. = 7):				
Mean ± SD	617 ± 91	75.4 ± 11.6	1.26 ± 0.74	0.22 ± 0.12
Range	502–741	63.2–91.8	0.38–2.33	0.05–0.30

TABLE 3

LEUKOCYTE HEX ACTIVITIES TOWARD SULFATED AND UNSULFATED FLUOROGENIC SUBSTRATES

GENETIC STATUS	HEX ACTIVITY (nmol hr $^{-1}$ mg $^{-1}$)			
	MU-β-GlcNAc	MU-β-GalNAc	MU-β-GlcNAc-6-S	MU-β-GalNAc-6-S
Noncarriers (no. = 30):				
Mean ± SD	1,400 ± 210	158 ± 24	254 ± 37	42.9 ± 6.2
Range	1,023–1,961	112–225	196–352	31.7–58.2
TSD-carriers (no. = 16):				
Mean ± SD	1,348 ± 218	154 ± 27	146 ± 22	24.0 ± 3.7
Range	870–1,705	100–196	97–183	16.7–30.4
TSD-patients (no. = 5):				
Mean ± SD	1,257 ± 187	148 ± 26	2.8 ± 1.1	0.44 ± 0.19
Range	1,060–1,550	131–190	1.6–4.1	0.31–0.68

TABLE 4

PERCENT HEX A IN INDIVIDUAL SERA AS DERIVED FROM DIFFERENTIAL HEAT-INACTIVATION AND FROM THE RATIO OF ACTIVITIES TOWARD SULFATED AND UNSULFATED SUBSTRATES

GENETIC AND PHYSIOLOGIC STATUS	HEX A BY HEAT	HEAT-STABLE MU-β-GlcNAc	TOTAL MU-β-GlcNAc	TOTAL MU-β-GlcNAc-6-S	HEX A BY RATIO*
	%	nmol hr^{-1} ml^{-1}	nmol hr^{-1} ml^{-1}	nmol hr^{-1} ml^{-1}	%
Noncarrier (no. = 33):					
Mean ± SD	66.0 ± 3.7	232 ± 63	701 ± 132	130 ± 25	67.0 ± 3.9
Range	60.7–73.8	151–385	494–1,045	93–180	60.4–74.5
Noncarrier pregnant (no. = 11):					
Mean ± SD	42.0 ± 5.1	500 ± 73	1,309 ± 248	138 ± 20	38.9 ± 5.9
Range	35.5–53.2	396–644	1,024–1,880	109–178	30.2–49.2
TSD carrier (no. = 19):					
Mean ± SD	45.8 ± 5.6	309 ± 78	608 ± 67	71.4 ± 7.0	42.5 ± 4.9
Range	37.0–54.4	269–440	509–743	58.8–83.5	36.0–52.9
TSD carrier with thermolabile HEX B (no. = 1)	56.9	260	603	69.2	41.6
TSD patients (no. = 7):					
Mean ± SD	2.94 ± 1.61	598 ± 88	617 ± 91	1.26 ± 0.74	0.73 ± 0.38
Range	0.90–5.31	496–725	502–741	0.38–2.33	0.24–2.33
TSD patient with thermolabile HEX B (no. = 1)	24.3	489	646	2.00	1.12

* A factor of 3.62 (based on fractionated serum HEX A) was used for conversion of MU-β-GlcNAc-6-S activity to the corresponding MU-β-GlcNAc activity of HEX A.

DISCUSSION

Direct determination of HEX A with the HEX A-specific MU-β-GlcNAc-6-S and MU-β-GalNAc-6-S substrates appears to be a reliable means for diagnosis of TSD patients and detection of TSD carriers. These fluorogenic assays are more sensitive than the chromogenic assay using *p*-nitrophenyl derivative [10–12] but are less sensitive when compared with the activity toward unsulfated fluorogenic derivatives. HEX A activity toward the most commonly used MU-β-GlcNAc substrate is 3.5–3.8 times higher than the activity toward MU-β-GlcNAc-6-S, and 21–24 times higher than its activity toward MU-β-GalNAc-6-S. The usefulness of sulfated fluorogenic substrates has been demonstrated by Inui and Wenger [13] and by Bayleran et al. [14] for the *N*-acetylglucosamine derivative and by us for both the *N*-acetylglucosamine and N-acetylgalactosamine derivatives. These are one-step direct assays as opposed to the two-step indirect heat-inactivation method [8, 9], and they are simple and inexpensive as compared with assays using radiolabeled G_{M2} ganglioside as substrate [20].

Although no overlap was observed among individual carrier and noncarrier values in the samples examined in this study, assignment of genotype should not rely solely on the activity toward sulfated substrates. Direct determination of HEX activity toward unsulfated substrates will account for total HEX activity in the same specimen and allow the calculation of % HEX A from total HEX. This may help in interpretation of borderline results. In addition, the ratio of the rate of hydrolysis of sulfated and unsulfated substrates will allow the discrimination between Sandhoff (G_{M2} gangliosidosis type O) patients and TSD patients [10, 11, 13] and between activator-deficient and HEX A-defective variants of G_{M2} gangliosidosis type AB [12].

Higher % HEX A values were calculated in TSD patients by the heat-inactivation method, and this may be due to a slight inactivation of the heat-stable isozymes (HEX B and the intermediate forms). HEX activity toward the sulfated substrates is independent of the thermostability of HEX B and the intermediate forms, and no interference was observed in the presence of hereditary heat-labile HEX B [21, 22]. The TSD carrier and TSD patient with thermolabile HEX B had 53.2% and 1.5% of the mean noncarrier activity toward MU-β-GlcNAc-6-S, and the values of % HEX A as derived from the sulfated/unsulfated activity ratios were 41.6% and 1.1%, respectively. The genotypes are clearly defined by both values. In serum specimens obtained from noncarrier women during pregnancy, % HEX A by ratio was as unreliable as that obtained by the heat-inactivation method. However, HEX activities measured directly with the sulfated substrates were clearly within the noncarrier range. Extended studies should be carried out in patients and carriers of various G_{M2} gangliosidoses to further evaluate the usefulness of these sulfated fluorogenic substrates.

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