Purification and Chemical Characterization of Human Hexosaminidases A and B

By JO ELLEN S. LEE and AKIRA YOSHIDA
*Division of Medicine, City of Hope Medical Center,
1500 E. Duarte Road, Duarte, CA 91010, U.S.A.*

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N-Acetyl- β -hexosaminidases A and B were purified to homogeneity from human placenta. In the initial step of purification, the enzymes were adsorbed on concanavalin A-Sepharose 4B and eluted from the column with α -methyl D-mannoside. Subsequent purification steps included DEAE-cellulose column chromatography, QAE-Sephadex [diethyl-(2-hydroxypropyl)aminoethyl-Sephadex] column chromatography, Sephadex G-200 gel filtration and preparative disc polyacrylamide-gel electrophoresis, followed by another QAE-Sephadex chromatography for the hexosaminidase A preparation, and DEAE-cellulose column chromatography, calcium phosphate gel chromatography, Sephadex G-200 gel filtration, QAE-Sephadex chromatography and CM-cellulose chromatography for the hexosaminidase B preparation. The purified preparations, particularly hexosaminidase A, had significantly higher specific enzyme activities than previously reported. The preparations moved on polyacrylamide-gel electrophoresis as single protein bands, which also stained for enzyme activity. Sedimentation-equilibrium centrifugation indicated homogeneous dispersion of the enzymes, and the molecular weight was estimated as about 110000 for both enzymes. Complete amino acid and carbohydrate compositions of the two isoenzymes were determined, and, in contrast with previous suggestions, no sialic acid was found in the enzymes.

N-Acetyl- β -D-hexosaminidase (EC 3.2.1.30, hereafter termed hexosaminidase) is an enzyme which catalyses the hydrolysis of the terminal *N*-acetylglucosaminyl or *N*-acetylgalactosaminyl moieties from glycolipids and polysaccharides (Weissman *et al.*, 1964; Sandhoff, 1970; Stirling, 1974) or from synthetic substrates in which the amino sugar is linked to a chromophoric group (Leaback & Walker, 1957, 1961). The enzyme exists in human and other mammalian tissues in two major isoenzymic forms, A and B, which differ from each other in their electrophoretic mobility, heat stability (Robinson & Stirling, 1968) and kinetic properties (Srivastava *et al.*, 1974b). Absence of the enzyme is associated with the inherited GM₂-ganglioside-storage diseases in man (Sandhoff, 1969), i.e. Tay-Sachs disease, in which hexosaminidase A activity is absent, and Sandhoff disease, in which both isoenzymes are missing.

Hexosaminidases A and B have been purified to homogeneity from human placenta in this laboratory. The finding that the enzymes were effectively adsorbed on to and eluted from concanavalin A-Sepharose 4B (Beutler *et al.*, 1975) facilitated the purification procedures. Moreover, hexosaminidase A with a substantially higher specific enzyme activity could be obtained by the present procedures. Here we report an improved purification method, and the molecular

weight and complete amino acid and carbohydrate composition of the two isoenzymes.

Materials and Methods

Human placentae were obtained from a local hospital and stored at 4°C for up to 24 h before use. 4-Methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside was purchased from Research Products International Corp., Elk Grove Village, IL, U.S.A. Concanavalin A-Sepharose and QAE-Sephadex [diethyl-(2-hydroxypropyl)aminoethyl-Sephadex] were purchased from Pharmacia, Uppsala, Sweden. Sephadex G-200 was from Sigma, St. Louis, MO, U.S.A., DEAE-cellulose (DE-22) and CM-cellulose (CM-52) were from Whatman, Maidstone, Kent, U.K., and fluorescamine (Fluram) was from Roche Diagnostics, Nutley, NJ, U.S.A. All other reagents used were of analytical grade.

Hexosaminidase activity was assayed by the method of Srivastava *et al.* (1974a). One unit of enzyme activity corresponds to 1 μ mol of substrate cleaved/min at 37°C. Protein determinations were done by either the method of Lowry *et al.* (1951) or the method of Udenfriend *et al.* (1972). Crystalline bovine serum albumin was used as the standard for both methods.

Polyacrylamide-disc-gel electrophoresis was performed by the method of Davis (1964); the gels were

stained for protein by using Coomassie Blue, and for enzyme activity by using 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside.

Results

Purification

The first three steps of the purification procedure were taken from Beutler *et al.* (1975). The various steps are outlined in Table 1.

Human placenta were washed with cold water and cleaned of membranes and blood vessels. Then 8 kg of cleaned placenta was cut into small pieces and homogenized in 16 litres of water in a Waring blender for 8 min. All procedures were carried out at 4°C unless otherwise indicated.

The 16-litre homogenate was sonicated for 5 min, in a Branson sonifier model W-350 (output control 8, pulsed mode on 40% of time). The homogenate was then centrifuged at 27 500g for 30 min. The pellet was re-homogenized in 2 vol. of water, re-sonicated and re-centrifuged. The supernatants were mixed in a ratio of 9 vol. of supernatant to 1 vol. of 0.125M-sodium succinate buffer, pH 6.0, containing 5 mM-MgCl₂, 5 mM-MnCl₂, 5 mM-CaCl₂ and 1% NaN₃.

The diluted supernatant was pumped through a concanavalin A-Sepharose column (2.5 cm $\times$ 24.5 cm), equilibrated with 12.5 mM-sodium succinate buffer, pH 6, containing 0.5 mM-MgCl₂, 0.5 mM-MnCl₂, 0.5 mM-CaCl₂ and 0.1% NaN₃. The column was washed with 0.01 M-phosphate buffer (Na₂HPO₄/

KH₂PO₄), pH 7.0, containing 0.5 M-NaCl, followed by 0.01 M-phosphate buffer (Na₂HPO₄/KH₂PO₄), pH 7.0. The column was eluted with 0.01 M-phosphate buffer (Na₂HPO₄/KH₂PO₄), pH 7.0, containing 0.5 M- α -methyl D-mannoside. These steps were carried out at 25°C. The sample could not be applied in one application, as the column became overloaded. After enzyme elution, the column was regenerated by thorough washing with the equilibrating buffer and more sample was applied. Active fractions were pooled, concentrated by vacuum dialysis, and applied to a DEAE-cellulose column (DE-22; 2.5 cm $\times$ 40 cm), equilibrated with 0.01 M-phosphate buffer (Na₂HPO₄/KH₂PO₄), pH 7.0. The column was washed with 760 ml of the equilibration buffer, and 20 ml fractions were collected. The enzyme that was not adsorbed on the column was designated hexosaminidase B. The enzyme adsorbed on the column was eluted with a linear gradient of increasing NaCl concentration and decreasing pH. The gradient was formed by using 1 litre each of 0.01 M-phosphate buffer (Na₂HPO₄/KH₂PO₄), pH 7.0, containing no NaCl, and 0.01 M-phosphate buffer (Na₂HPO₄/KH₂PO₄), pH 6.0, containing 0.2 M-NaCl. The fractions containing enzyme activity (from 0.05 M-NaCl to 0.2 M-NaCl) were pooled and designated hexosaminidase A.

At this point, the purification procedures for hexosaminidases A and B differ. From this step on, all buffer solutions contained 1 mM-EDTA and 1 mM-2-mercaptoethanol. Phosphate buffers were a mixture of Na₂HPO₄ and KH₂PO₄ to give the pH specified at the stated concentration at 25°C. All columns were

Table 1. Purification of placental hexosaminidases A and B

Yield is calculated by using the total enzyme activity of the crude preparation (homogenate, step 1). The true yield, obtained by using the individual activity of hexosaminidase A and of hexosaminidase B instead of the combined activity, results in a final yield of 8% for hexosaminidase A and 7% for hexosaminidase B.

Fraction	Total enzyme activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1. Homogenate	13 000	373 $\times$ 10 ³	0.035		100
2. Supernatant	10 000	280 $\times$ 10 ³	0.036	1	77
3. Concanavalin A-Sepharose 4B	5700	2650	2.2	63	44
Hexosaminidase A					
4. DE-22	4000	660	6.1	174	31
5. QAE-Sephadex 1	2400	230	10	290	19
6. Sephadex G-200	~2000	57	35	1000	15
7. QAE-Sephadex 2	~2000	46	44	1250	15
8. Preparative disc-gel electrophoresis	1300	11	120	3400	10
9. QAE-Sephadex 3	750	5	150	4300	5.8
Hexosaminidase B					
4. DE-22	1800	290	6.2	180	14
5. Calcium phosphate	1100	85	13	370	5.2
6. Sephadex G-200	880	33	27	770	6.8
7. QAE-Sephadex	900	33	27	770	6.8
8. CM-52	300	1.45	207	6000	2.3

equilibrated with the starting buffer. Every step was followed by concentration by vacuum dialysis and dialysis against the new starting buffer.

Hexosaminidase A. The pooled hexosaminidase A fractions from the DE-22 column were passed through a QAE-Sephadex column (2.5 cm × 35 cm), equilibrated with 0.02 M-Tris/HCl, pH 8.0, containing 0.1 M-NaCl. The adsorbed enzyme was eluted between 0.20 M- and 0.27 M-NaCl by using a 2-litre linear gradient of 0.1–0.4 M-NaCl in 0.02 M-Tris/HCl, pH 8.0. The concentrated, active fractions were then placed on a Sephadex G-200 column (2.5 cm × 65 cm) and eluted with 0.02 M-phosphate buffer, pH 7.0. The enzyme was then eluted from a QAE-Sephadex column (2 cm × 34 cm) between 0.25 M- and 0.30 M-NaCl by using a 400 ml linear gradient of 0.20–0.35 M-NaCl in 0.01 M-Tris/HCl, pH 7.0. After preparative disc-gel electrophoresis, the enzyme was eluted once more from a QAE-Sephadex column (2 cm × 23 cm) between 0.25 M- and 0.30 M-NaCl by using an 800 ml linear gradient of 0.1–0.4 M-NaCl in 0.02 M-Tris/HCl, pH 8.0.

Hexosaminidase B. The pooled hexosaminidase B fractions from the DE-22 column were placed on a calcium phosphate gel column (2.5 cm × 10 cm) and eluted with a 500 ml linear gradient of 0.10–0.25 M-phosphate buffer, pH 6.8, followed by 750 ml of 0.25 M-phosphate buffer, pH 6.8. The active fractions (found between 0.193 M- and 0.250 M-phosphate) were then eluted from a Sephadex G-200 column (2.5 cm × 65 cm) with 0.02 M-phosphate, pH 7.0. The pooled active fractions were then placed on a QAE-Sephadex column (2 cm × 25 cm) and eluted between 0.090 M- and 0.155 M-NaCl by using an 800 ml linear gradient of 0–0.2 M-NaCl in 0.02 M-Tris/HCl, pH 8.0. The final purification step was a CM-cellulose column (CM-52, 1 cm × 30 cm); the enzyme was eluted between 0.038 M- and 0.056 M-NaCl by using a 600 ml linear gradient of 0–0.1 M-NaCl in 0.005 M-phosphate buffer, pH 6.0.

Homogeneity

Both hexosaminidases A and B migrated as one band on polyacrylamide-gel electrophoresis when stained for enzyme activity and protein (Plate 1). The results of sedimentation-equilibrium centrifugation also revealed homogeneous molecular-weight species for both enzymes.

Molecular-weight determination

The enzymes were dialysed for 3 days against 0.1 M-phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7, at 4°C, and the protein concentration was adjusted to 0.1 mg/ml. The molecular weights of hexosaminidases A and B were determined by the sedimentation-equilibrium method (Yphantis, 1964), by using Ray-

leigh interference optics in a Spinco model E centrifuge. Centrifugation was carried out at 23150 rev./min for 20 h at 20°C.

A plot of the log of fringe displacement versus radius (Yphantis, 1964), which was measured by a Nikon comparator, revealed a straight line for both isoenzymes, indicating homogeneity with respect to molecular weight. A molecular weight of 119000 ± 9750 was determined for hexosaminidase A, and hexosaminidase B had a mol.wt. of 112500 ± 19000 . These measurements utilized a partial specific volume of 0.730 for hexosaminidase A and 0.722 for hexosaminidase B as calculated from their amino acid and carbohydrate compositions (Cohen & Edsall, 1943; McMeekin & Marshall, 1952).

Amino acid and carbohydrate composition

For determination of amino acid, neutral sugar and sialic acid contents, the enzymes were dialysed for 2 days against water, with several changes per day, and freeze-dried. The two isoenzymes were hydrolysed in constant-boiling HCl for 6, 24 and 72 h in sealed evacuated tubes. Cysteine determination involved performic acid oxidation of a small sample of each isoenzyme, following by hydrolysis for 20 h in constant-boiling HCl (Schram *et al.*, 1954). Tryptophan was determined from the tyrosine:tryptophan ratio, by the method of Goodwin & Morton (1946). Hexosaminidase A had a 3:2 ratio of tyrosine to tryptophan and hexosaminidase B had a 2:1 ratio. Hexosamine concentration (*N*-acetylglucosamine) was determined in the amino acid analysis, giving results of 2.45% for hexosaminidase A and 3.4% for hexosaminidase B after 6 h hydrolysis. Neutral sugars (mannose, glucose) were determined by the method of Winzler (1955). Hexosaminidase A contained 2.5% neutral sugar, and hexosaminidase B had 5.4%. Sialic acid was determined by the thiobarbituric acid method of Warren (1959). No sialic acid was found in either isoenzyme. This test can also detect 2-deoxyglucose, but none was found.

The amino acid and carbohydrate compositions of human hexosaminidases A and B are presented in Table 2. The number of amino acid and carbohydrate residues per subunit, also given in Table 2, was determined from the amino acid and carbohydrate compositions, by assuming an $\alpha_2\beta_2$ structure for hexosaminidase A and a β_4 structure for hexosaminidase B (Beutler *et al.*, 1976). A subunit mol.wt. of 27000 was used for the α and β subunits (Beutler *et al.*, 1976).

Discussion

Both hexosaminidases A and B were adsorbed on a concanavalin A-Sepharose 4B column and the enzymes were eluted from the column by α -methyl mannoside. This initial step alone increased the

Table 2. *Amino acid and carbohydrate composition of human placental hexosaminidases A and B*

Amino acid analysis was determined on a JEOL JLC-6AH automatic amino acid analyser, by using the standard two-column method. Except where noted, the value is the average of 24 h and 72 h hydrolysis in 6M-HCl at 110°C. The total of individual amino acids and sugars is taken to be 100. Values for serine and threonine are extrapolated to zero time of hydrolysis. Half-cystine was determined as cysteic acid in performic acid-oxidized protein. The recovery of cysteic acid is assumed to be 95%. Values for valine and isoleucine are for 72 h hydrolysis. The value for tyrosine is for 24 h hydrolysis. Tryptophan was measured by the method of Goodwin & Morton (1946). The value for *N*-acetylglucosamine is for 6 h hydrolysis in 3M-HCl. Neutral sugars were determined by the method of Winkler (1955), and sialic acid by the thiobarbituric acid method of Warren (1959). The numbers of residues/subunit were calculated from the amino acid and carbohydrate composition, assuming a subunit mol.wt. of 27000 for both α and β subunits and a molecular structure of $\alpha_2\beta_2$ for hexosaminidase A and of β_4 for hexosaminidase B (Beutler *et al.*, 1976). Values were rounded off to the nearest whole number.

Amino acid	Content (%)		Content (residues/subunit)	
	A	B	α	β
Aspartic acid	8.70	8.35	22	20
Threonine	4.68	4.86	12	14
Serine	5.08	6.44	12	21
Glutamic acid	11.17	10.00	27	22
Proline	4.79	4.23	16	12
Glycine	3.22	3.93	13	19
Alanine	3.13	3.54	11	14
Half-cystine	1.46	1.57	4	4
Valine	5.48	4.85	17	14
Methionine	2.22	1.96	6	4
Isoleucine	4.48	3.99	12	10
Leucine	8.78	7.90	23	20
Tyrosine	5.68	5.79	10	10
Phenylalanine	6.09	5.32	13	10
Lysine	5.32	6.26	9	14
Histidine	3.01	4.12	4	8
Arginine	7.46	4.79	18	9
Tryptophan	4.33	3.30	8	5
<i>N</i> -Acetyl-glucosamine	2.45	3.40	2	4
Neutral sugars	2.5	5.40	0	8
Sialic acid	0	0	0	0
Total	100	100	239	242

specific activity about 60-fold and facilitated the subsequent purification procedures. The preparations obtained by the present method appeared to be homogeneous on the basis of several physicochemical criteria, e.g. a single protein and enzyme band on polyacrylamide-gel electrophoresis (Plate 1), homogeneous dispersion in sedimentation equilibrium and

a single and symmetrical sedimentation boundary in analytical ultracentrifugation (Beutler *et al.*, 1976).

The specific activity of hexosaminidase A (about 150 units/mg) prepared by the present method is significantly higher than that of the previous preparation (87 units/mg) (Srivastava *et al.*, 1974a), whereas the specific activity of the present hexosaminidase B preparation and that of the previous preparation (by Method II of Srivastava *et al.*, 1974a) are similar. Despite the difference of specific enzyme activity, the molecular weight and amino acid composition of the present and previous preparations of hexosaminidase A are similar. Also, no impurities were detected in the previous preparation by polyacrylamide-gel electrophoresis. Therefore it is likely that the previous hexosaminidase A preparation was homogeneous, but that its activity was perhaps decreased, owing to partial inactivation.

Complete analysis of the amino acid and carbohydrate contents of homogeneous hexosaminidase A and B was accomplished in the present work (Table 2). In contrast with the frequently proposed idea (for example, see Robinson & Stirling, 1968; Goldstone *et al.*, 1971; Murphy & Craig, 1974), no sialic acid was detected in either enzyme. Hexosaminidase A has been shown to be a heterotetramer (Beutler & Kuhl, 1975), presumably with the structure $\alpha_2\beta_2$ (Beutler *et al.*, 1976), consisting of two similar-sized subunits with mol.wt. of about 27000, and hexosaminidase B is a homotetramer, presumably β_4 . Since hexosaminidase A contains approx. 2.5% hexose and hexosaminidase B contains approx. 5.4% hexose, the neutral sugars appeared to be limited to the β subunit. One can predict that hexosaminidase S, which is a homotetramer consisting of α subunits, is not adsorbed (or poorly adsorbed) on concanavalin A-Sepharose 4B. Indeed, this prediction has been verified by using liver from a patient with Sandhoff disease which contained no hexosaminidase A or B activities and low hexosaminidase S activity.

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EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of hexosaminidases A and B

Purified hexosaminidases A and B were tested for homogeneity on 7.5% acrylamide gels by the method of Davis (1964) (see the text for details). (1) Hexosaminidase B stained for protein; (2) hexosaminidase A stained for protein; (3) hexosaminidase B stained for enzyme activity with 4-methylumbelliferyl β -D-N-acetylglucosaminide as substrate; (4) hexosaminidase A stained for enzyme activity with the same substrate as in (3).

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