

pH Optima. Arylsulfatase A and B have acidic pH optima characteristic of lysosomal hydrolases. The exact pH maximum is unique for each substrate and optima for arylsulfatase B substrates span 2 pH units.³

Inhibitors. Arylsulfatases A and B are type II sulfatases, being inhibited by phosphate, sulfite, and sulfate, but not by cyanide ions. Arylsulfatase A is more sensitive to certain thiol reagents than arylsulfatase B and silver ions have been used to differentiate the enzymes.⁵

Stability. Both arylsulfatase A and B are remarkably stable. Tissue samples have been frozen for years without apparent loss of activity. Extended autolysis has been employed in the preparation of these enzymes from several sources. Procedures ranging from pH 3 to 9 have been employed with either enzyme. The thermal stability of arylsulfatase B is somewhat greater than that of the A enzyme; half inactivation times at 60° being 27 and 7 min, respectively, under comparable conditions.²¹ However, both enzymes become quite labile when the total protein concentration is less than ~0.1 mg/ml, and it is advisable to make all enzyme dilutions with buffers containing 1 mg of fatty acid free BSA per milliliter.

Cofactors. There are no cofactors required for the action of the arylsulfatases with synthetic substrates. A heat-stable factor of M_r 21,000 has been purified which potentiates the activity of arylsulfatase A with cerebroside sulfate in the absence of taurodeoxycholate.²³ It is uncertain whether this factor interacts with enzyme, substrate, or both.

Biomedical Significance. Arylsulfatase A is deficient in metachromatic leukodystrophy, a neurodegenerative genetic disease with autosomal recessive inheritance. Arylsulfatase A is among several lysosomal hydrolases effected by a faulty cellular retention mechanism in I-cell disease. A deficiency of arylsulfatase B is associated with the Maroteaux-Lamy syndrome, a genetic condition involving severe bone and connective tissue malformations. Arylsulfatases A and B and several additional sulfatases are lacking in tissues of patients with multiple sulfatase deficiency. Arylsulfatase A and/or B have been suggested to be of significance in reproductive processes, inflammation, arthritis, and neoplastic growth.

²³ G. Fisher and H. Jatzkewitz, *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 605 (1975).

[59] Hexosaminidases A and B from Human Placenta

By BENJAMIN GEIGER and RUTH ARNON

Hexosaminidase (2-acetamido-2-deoxy- β -D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) is an enzyme capable of hydrolyzing

the β -glycosidic linkage between either *N*-acetylglucosaminyl or *N*-acetylgalactosaminyl residues and various aglycons. In animal tissues isozymes of hexosaminidase are found in the lysosomes, where they partake in the degradation of glycosphingolipids, including gangliosides.

In human tissues two major isozymes of hexosaminidase have been described, designated hexosaminidases A (acidic) and B (basic).¹ It has been shown that the hereditary deficiency in hexosaminidase A, or in both hexosaminidases A and B, results in a fatal metabolic disorder—namely, GM₂ gangliosidosis (Tay–Sachs disease and Sandhoff–Jatzkewitz disease, respectively)—which are manifested by a massive accumulation of GM₂ in the central nervous system.²

Enzymic Assay

Reagents

Sodium citrate buffer, 40 mM, pH 4.4

Substrate: 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide, solution of 0.1 mg/ml in the above citrate buffer, containing 1 mg of bovine serum albumin (BSA) per milliliter

Glycine–NaOH buffer, 0.2 M, pH 10.4

Procedure: Enzymic Assay. Solutions containing hexosaminidase activity are diluted in the citrate buffer, pH 4.4, at 0°, and 0.1 ml aliquots are pipetted into duplicate tubes. The substrate solution (0.2 ml) is added, and the reaction mixture is incubated for 10 min at 37°. The reaction is terminated by the addition of 3 ml of the glycine–NaOH buffer, and the fluorescence of the reaction product, methylumbelliferone, is recorded in a fluorometer, using wavelengths 360 nm for excitation and 450 nm for emission. In our experience, a Turner fluorometer Model 110, equipped with 7-60 primary filter and filters Nos. 2A and 48 as secondary filters, was used with satisfactory results.

Product calibration curve is performed with several concentrations of freshly prepared methylumbelliferone solution in the glycine–NaOH buffer.

Units and Specific Activity. One unit of enzyme activity is defined as the amount of enzyme that will hydrolyze one micromole of the substrate per minute under the reaction conditions specified above. Specific activity is defined as number of enzymic units per milligram of protein.

¹ D. Robinson and J. L. Stirling, *Biochem. J.* **107**, 321 (1968).

² K. Sandhoff, K. Harzer, W. Wässle, and H. Jatzkewitz, *J. Neurochem.* **18**, 2469 (1971).

Purification of Human Placental Hexosaminidases A and B

Preparation of Sepharose-Bound Concanavalin A

Concanavalin A (Con A) ($\times 2$ crystallized in saturated NaCl solution, Miles-Yeda, Israel) is dialyzed for 1 hr at 20° against 0.1 M NaHCO₃ prior to binding to the CNBr-activated Sepharose: Sepharose 4B (Pharmacia, Sweden) is activated by CNBr (100 mg per gram of settled gel) for 8 min at 10°–15°, pH 11.0–11.5. The rinsed gel is mixed with the dialyzed Con A (5–8 mg per gram of gel). After 16 hr at 4° (with gentle stirring), the Con A-bound Sepharose is rinsed and kept at 4° in phosphate-buffered saline (PBS) until use. Yield of Con A binding under these conditions is usually about 90%.

Preparation of Sepharose-bound 2-Acetamido-N-(ϵ -aminocaproyl)-2-deoxy- β -D-glucopyranosylamine (Seph-CNAG)

The ligand (CNAG) used for the affinity chromatography of hexosaminidases is prepared according to Lis *et al.*³ This ligand inhibits the hydrolysis of 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide by human hexosaminidases A and B in a competitive manner with a K_i value of 1.57 mM and 1.55 mM for the A and the B isozymes, respectively.⁴ Cyanogen bromide-activated Sepharose 4B was mixed with the ligand in 0.1 M NaHCO₃ (5 mg of ligand per gram of settled gel) and maintained for 16 hr at 4°. The resultant gel is rinsed and allowed to react with 1 M ethanolamine at pH 8.0 for 2 hr to block unreacted groups. The amount of bound ligand is assessed by quantitative determination of ϵ -aminocaproic acid released from the column after acid hydrolysis. A typical value is 0.625 μ mol of ligand bound per 1 g of gel.

Purification Steps

Extraction of Hexosaminidases from Placentas. Human placentas obtained immediately after delivery are kept frozen at –20° until use. The thawed (or fresh) placentas are rinsed with tap water to remove excess blood, and homogenized in 4 volumes of ice-cold 10 mM phosphate buffer, pH 6.0, in a Waring commercial blender, for 3 min at top speed. The homogenate is centrifuged to remove the coarse particles (for large-scale preparation a Sharples continuous-flow centrifuge is recommended) and

³ H. Lis, R. Lotan, and N. Sharon, this series Vol. 34 [32].

⁴ B. Geiger, Y. Ben-Yoseph, and R. Arnon, *FEBS Lett.* **45**, 276 (1974).

frozen at -20° , unless further processed immediately. Recentrifugation at 16,700 *g* for 30 min at 4° is performed just before chromatography on Sepharose-Con A.

Chromatography on Sepharose-Con A. Clear placental homogenate (5–10 liters) is passed through Sepharose-Con A column (200 ml bed volume) at 4° at a flow rate of about 10 ml/min. The column is subsequently washed at 20° with 10 mM phosphate buffer, pH 6.0, and finally with PBS. The enzymically active peak is eluted with 10% α -methylglucoside in 0.5 M NaCl, concentrated in a hollow-fiber concentrator-dialyzer (Amicon, Holland) equipped with H1DP10 membrane cartridge, and dialyzed against 10 mM phosphate buffer, pH 6.0. Any precipitate formed during the dialysis is centrifuged off. The final volume obtained in this step is 2–5% of that of the starting solution.

Affinity Chromatography on Sepharose-CNAG. The affinity chromatography step is performed at 4° . Hexosaminidase solution obtained from the Con A column (about 1 liter) is loaded on Sepharose-CNAG column (120-ml bed volume). The gel is rinsed sequentially with 10 mM phosphate buffers of pH 6.0 and 7.0, followed by elution of the enzyme with 10 mM phosphate buffer of pH 8.2. The fractions under the peak of enzymic activity are pooled, concentrated on PM-30 Diaflow membrane (Amicon, Holland), and dialyzed against 10 mM phosphate buffer, pH 6.0.

Ion-Exchange Chromatography on DEAE-Cellulose. The enzymically active fraction obtained from the CNAG column is chromatographed on a DEAE-cellulose column (DE-52, Whatman, U.S.A., 80–100-ml bed volume) preequilibrated with 10 mM phosphate buffer pH 6.0. Hexosaminidase B is eluted with the equilibrating buffer, whereas hexosaminidase A, which adsorbs to the column, is subsequently eluted by 1 liter of linear salt gradient (0 to 0.2 M NaCl) in the phosphate buffer.

Hexosaminidase B is concentrated by vacuum dialysis or by ultrafiltration through a PM-30 membrane. Hexosaminidase A peak is concentrated similarly and dialyzed against 40 mM citrate buffer pH 4.4. Any precipitate formed during dialysis is centrifuged off.

Chromatography of Hexosaminidase A on CM-Cellulose. The concentrated hexosaminidase A is further purified on a column of CM-cellulose (CM-52, Whatman U.S.A., 50-ml bed volume), preequilibrated with 40 mM citrate buffer pH 4.4. The peak of pure hexosaminidase A, eluted with 0.5 M NaCl in the citrate buffer, is concentrated, dialyzed against PBS, and stored at -20° or -80° in small aliquots.

Gel Filtration of Hexosaminidase B on Sephadex G-150. The concentrated hexosaminidase B obtained from the DEAE-cellulose column, in a volume of 2–3 ml, is chromatographed at 4° on Sephadex G-150 (Phar-

TABLE I
PURIFICATION OF PLACENTAL HEXOSAMINIDASES A AND B

Preparation	Specific activity ^a (m units/mg protein)	Purification (fold)	Recovery (%)
<i>Hexosaminidase A</i>			
Homogenate	4.6	1	100
Seph-Con A eluate	101	22	65
Seph-CNAG eluate	2254	490	62
DEAE-cellulose	13980	3040	53
CM-cellulose	23300	5100	41
<i>Hexosaminidase B</i>			
Homogenate	3.9	1	100
Seph-Con A eluate	89.7	23	65
Seph-CNAG eluate	2028	520	61
DEAE-cellulose	14200	3640	52
Sephadex G-150	22600	5800	39

^a Differential determination of the two isozymes was carried out by both heat and pH inactivations.

macia, 100–150 ml bed volume), preequilibrated with PBS, at a rate of 10–12 ml/hr. The fractions with enzymic activity, located in the first of the two protein peaks eluted from the column, are pooled, concentrated, and stored frozen in small aliquots at -20° or -80° .

The extent of purification during the various steps in a typical preparation is given in Table I.

The purity of the two isozymes of hexosaminidase, prepared according to the above procedure, was established by sedimentation velocity run in the analytical ultracentrifuge and polyacrylamide gel electrophoresis in the presence or in the absence of SDS.

Properties of Hexosaminidases A and B

Stability

On storage at -80° or -20° , both A and B isozymes do not show any apparent loss of activity for up to 6 months. At temperatures of 37° and above, some sensitivity to heat is detected, hexosaminidase A showing lower stability than hexosaminidase B. At 50° under given conditions,^{1,5}

⁵ S. Okada and J. S. O'Brien, *Science* **165**, 698 (1969).

hexosaminidase A loses its entire activity within 90–100 min, whereas hexosaminidase B stays fully active. This serves as the basis for the differential determination of the two hexosaminidases.^{5,6} At 60° and above both isozymes are inactivated.

Both hexosaminidases are stable in neutral or slightly basic solutions. However, at low pH values, such as 2.8, hexosaminidase A is inactivated, whereas hexosaminidase B does not lose activity.

Molecular Properties

The physical, chemical, and enzymic properties of the two isozymes of hexosaminidase, as well as their molecular subunits, are given in Table II.

Information about other properties, including amino acid analysis, sulfhydryl and disulfide bridges content, sialic acid content, separation of the constituting polypeptide chains, was reported by Geiger and Arnon.⁷

Immunochemical Properties

Immunization. Antisera against hexosaminidases A and B are prepared by immunization of either rabbits or goats with 1.5–2.0 mg of the pure enzyme preparation, emulsified with complete Freund's adjuvant (Difco, U.S.A.), and injected intradermally at multiple sites. Booster injection can be given after 10–14 days; after this the animals are bled at weekly intervals.

The quality of the antisera is tested by the double immunodiffusion technique⁸ in agar gels (1.5% agarose in PBS), stained, after extensive rinsing, for enzymic activity, with a solution containing per milliliter 0.15 mg of naphthyl AS-BI *N*-acetyl- β -D-glucosaminide (Sigma, U.S.A.) and 1 mg of Fast garnet GBC salt (Sigma, U.S.A.) in 100 mM citrate buffer pH 4.4 at 37°.

Preparation of Anti-Hexosaminidase A Specific Antiserum. Antisera against both hexosaminidases A and B cross-react strongly with the heterologous isozymes; however, in double immunodiffusion anti-hexosaminidase A shows a spur formation with hexosaminidase A over the B isozyme.⁹ Hence, the "A-specific" antibodies are prepared by exhaustive adsorption:

Pure hexosaminidase B, or hexosaminidase B after the DEAE-

⁶ J. S. O'Brien, S. Okada, A. Chen, and D. L. Fillerup, *N. Engl. J. Med.* **283**, 15 (1970).

⁷ B. Geiger and R. Arnon, *Biochemistry* **15**, 3489 (1976).

⁸ Ö. Ouchterlony, *Acta Pathol. Microbiol. Scand.* **25**, 186 (1948).

⁹ Y. Ben-Yoseph, B. Geiger, and R. Arnon, *Immunochemistry* **12**, 221 (1975).

TABLE II
CHEMICAL AND ENZYMIC PROPERTIES OF PURE HEXOSAMINIDASES A AND B^a

Property	Hexosaminidase A	Hexosaminidase B
$S_{20,w}$	5.82	5.90
Molecular weight from sedimentation equilibrium analysis	99,200 ± 1,170	110,996 ± 778
Molecular weight from gel filtration on Sephadex G-200	100,000–103,000	107,000
Molecular weight of noncovalently bound subunits, obtained by sedimentation equilibrium analysis	51,992 ± 421	53,454 ± 561
Molecular weight of polypeptide chains of reduced and carborymethylated enzyme, obtained by sedimentation equilibrium analysis and gel electrophoresis	23,000–25,000	25,000
Isoelectric point	5.2	7.7
pH optimum	4.4–4.6	4.4–4.6
K_m value for MUF-GlcNAc ^b	$5.18 \times 10^{-4} M$	$5.18 \times 10^{-4} M$
K_m value for MUF-GalNAc ^c	$1.12 \times 10^{-4} M$	$1.12 \times 10^{-4} M$
V_{max} value with MUF-GlcNAc (units/mg protein)	214.4	207.9
V_{max} value with MUF-GalNAc (units/mg protein)	28.6	27.7
Inhibition by <i>N</i> -acetylglucosamine	Competitive, $K_i = 3.7 \text{ mM}$	Competitive, $K_i = 4.0 \text{ mM}$
Inhibition by <i>N</i> -acetylgalactosamine	Competitive, $K_i = 0.40 \text{ mM}$	Competitive, $K_i = 0.41 \text{ mM}$

^a B. Geiger and R. Arnon, *Biochemistry* **15**, 3489 (1976); B. Geiger, R. Navon, Y. Ben-Yoseph, and R. Arnon, *Eur. J. Biochem.* **56**, 311 (1975).

^b MUF-GlcNAc-4-methylumbelliferyl *N*-acetyl β -D-glucosaminide.

^c MUF-GalNAc-4-methylumbelliferyl *N*-acetyl β -D-galactosaminide.

cellulose chromatography step, is coupled to CNBr-activated Sepharose 4B (1 and 5 mg, respectively, per gram of Sepharose). Aliquots of 100 ml of anti-hexosaminidase A are passed through the immunoabsorbent column containing about 15 mg of immobilized hexosaminidase B, which is then washed with PBS and regenerated with 8 *M* urea. For a complete

removal of the anti-B reactivity, about 15 repeated absorptions are required: the resultant specific anti-hexosaminidase A antiserum is concentrated by precipitation with ammonium sulfate (40% saturation), dissolution in a minimal volume of PBS, and dialysis against the buffer.

These two serological reagents can serve for differential immunological determination of A and B isozymes of hexosaminidase in the presence of each other, by sensitive techniques such as radial immunodiffusion and radioimmunoassay, as described in the following section.

Immunochemical Determination of Hexosaminidases A and B¹⁰

Radial Immunodiffusion. Solution of agarose (1.5% in PBS) mixed with an appropriate dilution of antiserum (according to the titer) is poured into plastic plates to yield a 2.0–2.4-mm agar layer. After complete gelification at 4°, wells with a diameter of 3 mm are punched at 10-mm intervals from each other, and 10- μ l samples of enzyme solution are applied to the wells, using a precision syringe (Hamilton 701 N). It is recommended that each sample be tested in several serial dilutions. Diffusion is allowed to proceed for 36 hr at 4°, after which the plates are extensively rinsed with PBS (48 hr) and stained for enzymic activity as mentioned above. The net areas of the precipitin rings are measured either directly from the plate, or after enlargement of their negative films. The total hexosaminidase (A + B) is determined in a gel containing the cross-reactive antiserum toward hexosaminidase B, whereas the gel containing the specific anti-A antibodies determines hexosaminidase A exclusively. The relative dilutions of the two antisera should be such as to give an identical size ring with the same concentration of hexosaminidase A. The technique yields best results when the size of the ring is within the range of 20–80 mm² net area (5–11 mm diameter).

Radioimmunoassay. Hexosaminidase A is labeled with ¹²⁵I, according to the lactoperoxidase method,¹¹ as follows: Enzyme solution (10 μ l of 1.5 mg/ml in PBS) is added to 10 μ l of 200 mM phosphate buffer, pH 7.2. Lactoperoxidase (5 μ l of 600 μ g/ml) is added to the mixture, followed by 1 mCi of carrier-free [¹²⁵I]Na in 10 μ l, and 5 μ l of 8.8 mM hydrogen peroxide. The reaction is allowed to proceed for 30 min at 37°, with an intermediate addition of H₂O₂. Cold PBS (200 μ l) is added and the solution is chromatographed on Sephadex G-25 (fine) for the removal of unbound iodine. The labeled enzyme is diluted in PBS containing 0.1% gelatin and stored at –20° until used.

¹⁰ B. Geiger, R. Navon, Y. Ben-Yoseph, and R. Arnon, *Eur. J. Biochem.* **56**, 311 (1975).

¹¹ J. J. Marchalonis, *Biochem. J.* **113**, 299 (1969).

Antigen-binding capacity of the various antibody preparations is performed as follows. Fifty microliters of serial dilutions of goat antiserum toward hexosaminidase B or specific anti-hexosaminidase A serum (in 2.5% normal goat serum in PBS) are added to 50 μ l of iodinated hexosaminidase A (10^4 cpm) in plastic tubes (2052, Nunc, Denmark). After 30 min at 37°, rabbit anti-goat immunoglobulin is added, in an amount sufficient for complete precipitation of all the goat antibodies. After 30 min at 37° and 16 hr at 4°, the precipitate is spun down, rinsed, and monitored for radioactivity.

In competition experiments, pure isozymes or biological specimens are allowed to compete with the binding of radiolabeled enzyme. Solutions containing 1–1000 ng of hexosaminidases A and/or B, in 50 μ l, are mixed with the radioactive hexosaminidase prior to the addition of antiserum. The assay of bound labeled enzyme then proceeds as described above. The results are expressed as percent inhibition of binding as a function of the concentration of unlabeled hexosaminidase. The differentiation between hexosaminidases A and B is according to the differential inhibitory activity in the assays with the cross-reactive and the A-specific antisera, respectively.

[60] endo- β -N-Acetylglucosaminidase D from *Diplococcus pneumoniae*

By TAKASHI MURAMATSU

endo- β -N-Acetylglucosaminidase D is a unique endoglycosidase found in the culture fluid of *Diplococcus pneumoniae*.¹ The enzyme cleaves di-N-acetylchitobiose linkage in asparagine-linked oligosaccharides² and has strict specificity with respect to the structure of oligomannosyl cores of the substrates.^{2–4} The endoglycosidase has been useful in structural

¹ T. Muramatsu, *J. Biol. Chem.* **246**, 5535 (1971).

² N. Koide and T. Muramatsu, *J. Biol. Chem.* **249**, 4897 (1974).

³ S. Ito, T. Muramatsu, and A. Kobata, *Biochem. Biophys. Res. Commun.* **63**, 938 (1975).

⁴ T. Tai, K. Yamashita, M. Ogata-Arakawa, N. Koide, T. Muramatsu, S. Iwashita, Y. Inoue, and A. Kobata, *J. Biol. Chem.* **250**, 8569 (1975).