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Purification and Properties of Human Kidney-Cortex Hexosaminidases A and B

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Hexosaminidases (EC 3.2.1.30) A and B from human kidney cortex were purified to homogeneity by using concanavalin A affinity chromatography, ion-exchange chromatography and gel filtration. The yield of homogeneous isoenzymes improved approx. 20-fold, giving preparations of hexosaminidases A and B with specific activities of about 200 and 325 units/mg of protein respectively. The kinetic and structural properties of kidney hexosaminidase isoenzymes were studied and compared with the hexosaminidase isoenzymes from human placenta. The amino acid composition of hexosaminidase A was significantly different from that of hexosaminidase B. In the event of success in developing enzyme-replacement therapy for Tay–Sachs and Sandhoff's diseases, this modified procedure can furnish larger amounts of homogeneous isoenzymes.

β-D-*N*-Acetylhexosaminidases (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) A and B are lysosomal hydrolases involved in the catabolism of GM2 ganglioside and GA2 globoside. The specificity of the isoenzymes towards these substrates is reflected in Tay–Sachs disease, in which hexosaminidase A is deficient and GM2 ganglioside is accumulated (Kolodny *et al.*, 1969; Okada & O'Brien, 1969; Hultberg, 1969; Sandhoff, 1969), and Sandhoff's disease, in which both isoenzymes are deficient and GM2 ganglioside along with GA2 globoside is accumulated (Sandhoff *et al.*, 1968; Sandhoff, 1969).

Both isoenzymes have been purified from microorganisms and mammalian tissues (Price & Dance, 1967; Mega *et al.*, 1970; Sandhoff & Wassle, 1971; Verpoorte, 1972; Wetmore & Verpoorte, 1972; Srivastava & Beutler, 1972; Carroll & Robinson, 1973; Srivastava *et al.*, 1974*a*; Tallman *et al.*, 1974). In earlier studies, we purified hexosaminidase A and hexosaminidase B to homogeneity from human placenta and studied their kinetic, structural and immunological properties (Srivastava *et al.*, 1974*a*,*b*; Srivastava & Beutler, 1974). Since the yield of hexosaminidase A and hexosaminidase B from our earlier purification (Method I, Srivastava *et al.*, 1974*b*) was of the order of 1–2%, we have improved the purification technique from human placenta and kidney, which results in a yield of about 36% and a final specific activity of more than twice that of the earlier preparations. Human kidney was chosen as the starting material for purification because it contains almost three times as much enzyme per g wet weight as liver and five times as much as placenta.

We (Cohen *et al.*, 1976) have demonstrated that hexosaminidase A, sequestered in liposomes and coated with heat-aggregated immunoglobulin G, is taken up by human leucocytes *in vitro* and is deposited in lysosomes. The potential of successful enzyme-replacement therapy in lysosomal storage diseases has been greatly enhanced as a result of these studies. Thus this improved purification technique, resulting in a high yield of enzyme, will be of great importance whenever enzyme-replacement therapy is developed. In addition, the availability of larger amounts of homogeneous hexosaminidases will facilitate detailed structural studies of these proteins.

Materials and Methods

4-Methylumbelliferyl β-D-*N*-acetylgalactosamide and glucosaminide analogues were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Ampholine was purchased from LKB Produkter AB, Bromma, Sweden. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were obtained from Whatman, Clifton, NJ, U.S.A. and concanavalin A insolubilized on beaded Sepharose, α-methyl mannoside and ECTEOLA-cellulose (epichlorhydrin triethanolamine-cellulose) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden. Normal human kidneys were obtained at post-mortem. The medulla was removed and the cortex was frozen at −20°C for several months until used. Unless otherwise specified, all purification steps were performed at 4°C.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Polyacrylamide-gel disc electrophoresis was performed by the method of Davis (1964) and the gels

were stained for protein and enzyme activity as described by Srivastava *et al.* (1974*a*). The molecular weight of hexosaminidase A and B was determined by Sephadex G-200 gel filtration by using glutathione peroxidase, aldolase, albumin and ribonuclease A as standards. The molecular weight was also determined by boundary ultracentrifugation as described previously (Srivastava *et al.*, 1974*a*). Subunit molecular weights were determined by sodium dodecyl sulphate/urea/β-mercaptoethanol/polyacrylamide-gel disc electrophoresis (Srivastava *et al.*, 1974*a*). Amino acid-composition analyses were obtained with a Beckman 119 analyser by single-column methodology on Durrum DC-6A resin. Temperature programming was used to separate glucosamine and galactosamine by using the above methodology.

Purification of hexosaminidases A and B

Human kidney cortex (2000g) was homogenized in water in a Waring Blendor to give a 20% (w/v) homogenate. It was further homogenized by using a Sorvall Omni-Mixer at 8000rev./min for 10min and incubated at 37°C for 2h. This kidney homogenate was also used for purification of γ-glutamyltranspeptidase (EC 2.3.2.2) (Miller *et al.*, 1976), for which heating the homogenate at 37°C was an essential first step. In a separate experiment the effect of heating the homogenate at 37°C for 2h on the ratio of total amount of hexosaminidase A and B was determined by column chromatography on DE-52 DEAE-cellulose. Since no significant change in the hexosaminidase A/B ratio was observed in the heat-treated homogenate, this step, which precipitates a large amount of protein from the homogenate, was retained for purification of hexosaminidases A and B. The homogenate was subsequently centrifuged at 10000*g* at 4°C for 1h and the supernatant was passed through a column (1.5cm×25cm) packed with concanavalin A insolubilized on beaded Sepharose, at 15ml/h. The column was pre-equilibrated with 20mM-potassium phosphate buffer, pH7.0, containing 2mM-$MgCl_2$ and -$MnCl_2$. After the column was saturated with the enzyme, it was washed with 100ml of equilibrating buffer containing 1M-NaCl. The enzyme was eluted from the column with 0.1M-α-methyl mannoside at 30ml/h. The column was regenerated by passing 300ml of the equilibrating buffer. The unadsorbed enzyme was recycled through the column and eluted as before. This process was repeated five times; approx. 1200–1500 units of enzyme were adsorbed and eluted from the column cycle. The enzyme activity was determined as described previously (Srivastava *et al.*, 1974*a*). The fractions containing enzyme activity were pooled, concentrated to about 400ml by using an Amicon ultrafiltration cell model 402 with a PM-10 membrane and dialysed for 48h against 4 litres of 10mM-potassium phosphate buffer, pH6.0, with three changes of the dialysing buffer.

The dialysed preparation was centrifuged at 10000*g* for 30min and the supernatant was passed through a DEAE-cellulose DE-52 column (2.5cm×30cm), equilibrated with 10mM-potassium phosphate buffer, pH6.0, containing 30mM-NaCl at 50ml/h. Under these conditions, hexosaminidase B did not bind to the column. The column was subsequently washed with the 600ml of equilibrating buffer and the elution of hexosaminidase A was performed with a linear gradient (1500ml) of 30–250mM-NaCl in 10mM-potassium phosphate buffer, pH6.0, at 50ml/h. The fractions containing hexosaminidase A activity were pooled, concentrated to about 80ml by using an Amicon ultrafiltration cell model 402 with a PM-10 membrane, and centrifuged at 10000*g* for 30min. The supernatant was passed through a Sephadex G-200 column (5.0×90cm) equilibrated with 10mM-potassium phosphate buffer, pH7.0, containing 0.1M-$(NH_4)_2SO_4$, at an upward flow rate of 60ml/h. The fractions containing hexosaminidase A activity were pooled and dialysed for 24h against 4 litres of 10mM-potassium phosphate buffer, pH7.5, containing 30mM-NaCl.

The dialysed supernatant was passed through the ECTEOLA-cellulose column (2.5cm×30cm) equilibrated with 10mM-phosphate buffer, pH7.5, containing 30mM-NaCl, at 40ml/h. The column was washed with 300ml of the equilibrating buffer and then hexosaminidase A was eluted with a 600ml linear gradient of 30–200mM-NaCl in 10mM-phosphate buffer, pH7.5. The fractions containing hexosaminidase A activity were pooled and concentrated by using Amicon ultrafiltration equipment and used for structural, kinetic and immunological studies.

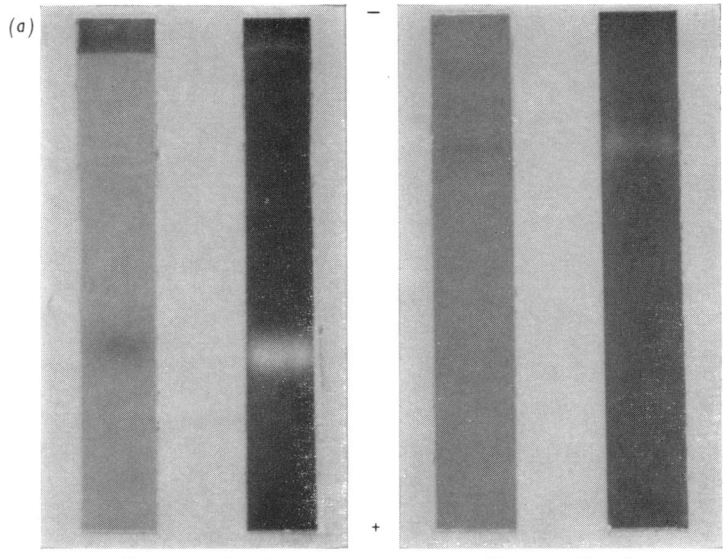
Purification of hexosaminidase B

The unadsorbed enzyme from the DEAE-cellulose DE-52 column was passed through a CM-cellulose CM-52 column (2.5cm×30cm, equilibrated with 10mM-potassium phosphate buffer, pH5.0) at 30ml/h. The column was washed with 300ml of the equilibrating buffer, and hexosaminidase B was eluted with a 2-litre linear gradient of 0–0.9M-NaCl in the equilibrating buffer. The fractions containing the enzyme activity were pooled, concentrated to 80ml by ultrafiltration, dialysed and passed through a Sephadex G-200 column as for hexosaminidase A. The fractions containing hexosaminidase B activity were pooled, dialysed against water and used for further studies.

Results

Purification of hexosaminidases A and B

The purification of hexosaminidases A and B is presented in Table 1. In the final purification step of



EXPLANATION OF PLATE 1

Polyacrylamide-gel disc electrophoresis of human kidney hexosaminidase A and hexosaminidase B

(*a*) Enzyme (6–8 mg of protein) was applied to polyacrylamide gels. Electrophoresis was performed in a discontinuous double-buffer system as described earlier (Davis, 1964; Srivastava *et al.*, 1974*a*). Gels were stained for protein with Coomassie Blue (left gel of each set) and for enzyme with 4-methylumbelliferyl β-D-*N*-acetylglucosaminide (right gel of each set) as described earlier (Srivastava *et al.*, 1974*a*). (*b*) Hexosaminidase A-overloaded gel. Hexosaminidase A (80 mg) was applied to polyacrylamide gels; electrophoresis and staining were carried out as described in (*a*). A small amount of hexosaminidase A used for this gel had been converted into hexosaminidase B on storage for about 3 months at 4°C. The left gel represents protein stain and the right gel represents enzyme stain.

Table 1. *Purification of hexosaminidases A and B from human kidney cortex*

Purification step	Activity (units/ml)	Activity (total units)	Protein (mg/ml)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Whole homogenate	0.83	9130	4.88	0.17	—	100
Supernatant after 37°C	0.88	9130	3.40	0.26	1.5	100
Concanavalin A	21.0	5476	6.12	3.43	20	60
Hexosaminidase A						
DEAE-cellulose DE-52	6.04	2739	0.47	12.9	76	30
Sephadex G-200	1.75	2524	0.02	87.5	515	28
ECTEOLA-cellulose	6.52	1808	0.03	204	1199	20
Hexosaminidase B						
DEAE-cellulose DE-52	4.08	2998	0.22	17.2	101	33
CM-cellulose CM-52	9.04	1898	0.15	60.3	355	21
Sephadex G-200	6.52	1472	0.02	326	1918	16

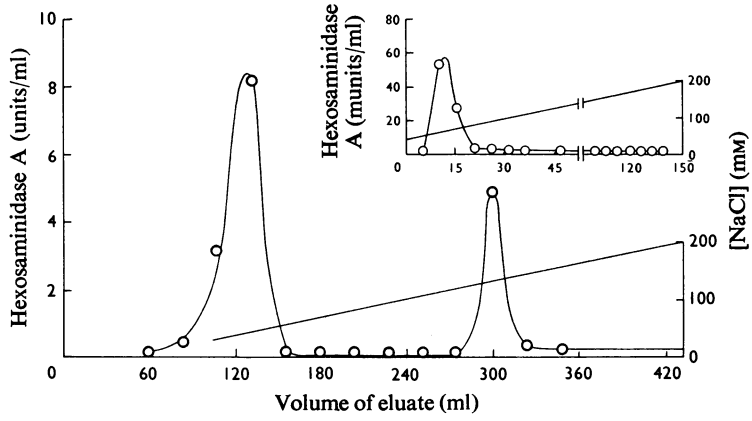


Fig. 1. *Purification of human kidney hexosaminidase A by ECTEOLA-cellulose column chromatography*
The experimental details are given in the text. The second peak of enzyme activity was subjected to rechromatography in a Pasteur-pipette column (6cm bed height). The inset shows the elution pattern of the rechromatography.

hexosaminidase A, by ECTEOLA-cellulose column chromatography, the enzyme was eluted in two peaks (Fig. 1), which have different electrophoretic mobilities on polyacrylamide gels. However, on rechromatography of the more anionic peak, most of the enzyme activity was eluted at the lower salt concentration (Fig. 1), corresponding to the salt concentration required to elute the less anionic peak. Thus it was concluded that on ECTEOLA-cellulose chromatography, the net anionic charge of hexosaminidase A was decreased, possibly owing to the loss of neuraminic acid.

Hexosaminidase A was purified about 1200-fold to a specific activity of about 204units/mg and hexosaminidase B was purified 1900-fold to a specific activity of about 326units/mg (Table 1). The total yield of both the isoenzymes is about 36%. Both hexosaminidase A and hexosaminidase B migrated as a single protein band on polyacrylamide-gel disc electrophoresis which stained for the enzyme activity (Plate 1*a*). To lessen the possibility of a minor contaminant in the purified preparations of hexosaminidase A and hexosaminidase B, the polyacrylamide gels were loaded with about 80μg of protein (10 times the usual amount). A scan of the overloaded gels at 280nm demonstrated the homogeneity of both hexosaminidase A and hexosaminidase B. An overloaded gel with hexosaminidase A is presented in Plate 1(*b*).

Neither hexosaminidase A nor hexosaminidase B lost significant enzyme activity at 4°C in 10mM-phosphate buffer, pH6.0, after about 1 month. Hexosaminidase B lost about 15% of enzyme activity in 4 months under similar conditions, and about 30% of hexosaminidase A activity was lost over 3 months at 4°C. Hexosaminidase B could be stored

frozen at −10°C without a substantial loss of enzyme activity for about 4 months, whereas a significant amount of hexosaminidase A was converted into hexosaminidase B on prolonged freezing and subsequent thawing or prolonged incubation at 4°C.

The mol.wt. of both hexosaminidase A and hexosaminidase B was found to be about 14000 by Sephadex gel filtration and about 100000 by ultracentrifugation. These results are similar to those of the placental isoenzymes, as reported previously (Srivastava *et al.*, 1974*b*). Hexosaminidase A dissociated into subunits of mol.wts. of about 18000 and 40000 on sodium dodecyl sulphate/urea/β-mercaptoethanol/polyacrylamide-gel disc electrophoresis, whereas hexosaminidase B dissociated into subunits corresponding to a mol.wt. of about 18000.

The isoelectric pH of hexosaminidase A and hexosaminidase B was 5.4 and 7.5 respectively. Both isoenzymes had an optimum pH of enzyme activity at about 4.4 with 4-methylumbelliferyl β-D-*N*-acetylgalactosaminide or -glucosaminide as substrate. For both of these substrates the K_m was about 0.5mM.

The amino acid compositions of both hexosaminidases A and B are presented in Table 2.

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

Values are given in residues per molecule, and are determined from time-course hydrolysis of 24, 48 and 96h and based on a mol.wt. of 100000 for both hexosaminidase A and B, and 10% and 8% carbohydrate respectively (S. K. Srivastava & A. Yoshida, unpublished work). Numbers in parentheses are nearest whole numbers (average residues). Values for half-cystine and glucosamine are tentative only, because the material was insufficient for specialized hydrolysis. n.d., Not determined.

Amino acid	Hexosaminidase A	Hexosaminidase B
Aspartic acid	81.2 (81)	83.9 (84)
Threonine	68.1 (68)	50.8 (51)
Serine	85.0 (85)	59.8 (60)
Glutamic acid	84.5 (84–85)	88.3 (88)
Proline	49.8 (50)	61.1 (61)
Glycine	63.2 (63)	67.6 (67–68)
Alanine	66.0 (66)	51.2 (51)
Half-cystine	13.3 (13)	4.5 (4–5)
Valine	70.4 (70–71)	53.7 (54)
Methionine	7.6 (7–8)	11.2 (11)
Isoleucine	29.5 (29–30)	34.9 (35)
Leucine	78.1 (78)	78.2 (78)
Tyrosine	20.7 (21)	35.7 (36)
Phenylalanine	32.0 (32)	44.6 (44–45)
Lysine	46.5 (46–47)	48.8 (49)
Histidine	14.3 (14)	21.4 (21–22)
Arginine	30.8 (31)	35.3 (35)
Glucosamine	8.5 (9)	trace
Tryptophan	n.d.	n.d.

Discussion

A significant increase in the total yield of hexosaminidase A and hexosaminidase B and an increase in specific activity of these isoenzymes over the previously published purification procedures has been achieved. The yield of homogeneous hexosaminidases in the current scheme of purification is about 20-fold higher than in the method reported previously (Srivastava *et al.*, 1974*a*). The specific activities of both hexosaminidase A and hexosaminidase B obtained by the present procedure are more than 2-fold greater than those reported in Method I of the earlier studies. On the other hand, the specific activity of only hexosaminidase A was doubled, whereas that of hexosaminidase B is less elevated as compared with the values obtained by Method II in Srivastava *et al.* (1974*a*). The improved yield and increased specific activities may be due to the elimination of the freeze-drying, $(NH_4)_2SO_4$ precipitation and isoelectrofocusing steps (Srivastava *et al.*, 1974*a*) and the inclusion of the concanavalin A affinity-chromatography step.

The molecular weights of hexosaminidase A and hexosaminidase B from human kidney were similar to that of placental enzymes. During sodium dodecyl sulphate / urea / β-mercaptoethanol / polyacrylamide-gel disc electrophoresis, kidney hexosaminidase B, like placental hexosaminidase B, almost completely dissociated into a single polypeptide of mol.wt. about 18000. However, under identical conditions, kidney hexosaminidase A exhibited an additional protein band corresponding to a mol.wt. of about 40000, along with the 18000-mol.-wt. protein. In our previous studies (Srivastava *et al.*, 1974*b*), hexosaminidase A dissociated into at least three protein bands corresponding to mol.wts. of 18000, 35000 and 55000, which probably represented monomers, dimers and trimers respectively. Since it is difficult to distinguish between proteins with mol.wts. of 35000 and 40000, it appears that the additional protein band represents a dimer, indicating that the complete dissociation of hexosaminidase A does not occur under these conditions. Thus like the placental isoenzymes, the kidney isoenzymes also appear to be hexamers of subunits with mol.wts. of 17000–18000. Immunological studies indicated that kidney hexosaminidase A and hexosaminidase B share a common subunit (β) and that hexosaminidase A has an additional subunit (α) not present in hexosaminidase B (Srivastava & Beutler, 1973, 1974; Srivastava *et al.*, 1976). Therefore the kidney hexosaminidase A and hexosaminidase B, like the placental enzymes, can be represented as $(\alpha\beta)_3$ and $(\beta\beta)_3$ respectively.

These results, however, are not in agreement with the reports (Beutler *et al.*, 1976; Lee & Yoshida, 1976; Geiger & Arnon, 1976) in which placental hexosaminidases A and B have been assigned a tetra-

meric structure consisting of subunits of 25000–28000 mol.wt. These investigators (Beutler *et al.*, 1976; Lee & Yoshida, 1976) used the method of Segrest & Jackson (1972) to determine the subunit molecular weight of glycoproteins. In this method the protein is incubated with 1% sodium dodecyl sulphate and 10mM-dithiothreitol for 1h at 37°C, pH 7.1; 8M-urea is added to the sample just before polyacrylamide-gel disc electrophoresis to give final concentrations of 4M-urea and 0.5% sodium dodecyl sulphate. Using this method we also find the subunit mol.wt. of kidney and placental hexosaminidases A and B to be about 25000. This agrees with a tetrameric structure. However, incubation of the proteins with 1% sodium dodecyl sulphate in 140mM-β-mercaptoethanol, pH7.4, and 6M-urea or -guanidine hydrochloride for 5h at 37°C (Srivastava *et al.*, 1974*b*) and subsequent polyacrylamide-gel disc electrophoresis dissociated hexosaminidase isoenzymes into subunits of mol.wt. 17000–18000. This indicates that these isoenzymes are hexamers rather than tetramers. The differences in the apparent molecular weight are probably due to the differential binding of sodium dodecyl sulphate with the glycoprotein under different experimental conditions. There are numerous examples of such discrepancies in the apparent molecular weight of glycoprotein subunits when determined by sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis under different conditions (Segrest & Jackson, 1972).

The subunit composition of glycoproteins can also be determined by amidation and cross-linking by dimethyl suberimidate (Klotz *et al.*, 1970; Davies & Stark, 1970). We have used this method to determine the number of subunits in human placental hexosaminidase A. Our preliminary results (J. E. Wiktorowicz & S. K. Srivastava, unpublished work) indicate that hexosaminidase A has at least six subunits.

The amino acid compositions of kidney hexosaminidase A and hexosaminidase B are significantly different from each other, and are somewhat different from their placental counterparts reported earlier (Srivastava *et al.*, 1974*b*). The differences between the kidney and placental enzymes are at present unexplained. These differences may be due to different carbohydrate contents of the enzymes giving rise to variations in the amino acid analysis due to humin formation. Alternatively, the enzymes from the different tissues may represent slightly different cleavage products of as yet undetermined precursor forms.

The improved method of purification of hexosaminidases reported here giving higher specific activities will be of great significance in providing larger quantities of the homogeneous enzyme for enzyme-replacement therapy in Tay–Sachs and Sandhoff's diseases as well as providing ample protein for detailed structural analyses.

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