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Purification of Normal Human Urinary *N*-Acetyl-β-hexosaminidase A by Affinity Chromatography

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N-Acetyl-β-hexosaminidase A was purified 1000-fold from human urine by chromatography on DEAE-Sephadex followed by concanavalin A–Sepharose affinity chromatography. The optimal pH range was 4.4–4.5 for both the *N*-acetylglucosamine and *N*-acetylgalactosamine derivatives. The K_m values were 0.51 mM and 0.28 mM respectively for the *N*-acetylglucosamine and *N*-acetylgalactosamine derivatives. The glycoprotein nature of the urinary enzyme was established by its affinity towards concanavalin A as well as by the presence of sialic acid, galactose, glucose, mannose and hexosamines in the molecule.

N-Acetyl-β-D-hexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30), an enzyme with both β-galactosaminidase and β-glucosaminidase activity, is believed to be involved in the normal sequential degradation of glycolipids, glycoproteins and glycosaminoglycans in lysosomes (Nisizawa & Hashimoto, 1970). The enzyme is widely distributed. It has been purified to varying degrees from different sources, including human spleen (Robinson & Stirling, 1968), pig epididymis (Findlay & Levvy, 1960), calf brain (Frohwein & Gatt, 1967), *Aspergillus niger* (Bahl & Agarwal, 1969), *Aspergillus oryzae* (Mega *et al.*, 1970), hen oviduct (Tarentino & Maley, 1971), and human liver (Sandhoff & Waessle, 1971; Carroll & Robinson, 1973). The enzyme has been crystallized from jackbean meal (Li & Li, 1970).

Robinson & Stirling (1968) first showed that hexosaminidase activity could be attributed to two components A and B, which could be resolved by starch-gel electrophoresis or by anion-exchange chromatography, or distinguished by their different heat stabilities. Interest in this enzyme was greatly stimulated by the demonstration of a specific deficiency of component A in tissues from patients with Tay–Sachs disease (Okada & O'Brien, 1969; Hultberg, 1969) and a deficiency of both components in a variant of the disease (Sandhoff, 1969). In addition to the major A and B forms, at least two minor components were found to be present in the tissue extracts and serum (Sandhoff, 1968; Young *et al.*, 1970; Murphy & Craig, 1972; Ikonne & Ellis, 1973). Dance and his co-workers (Dance *et al.*, 1969, 1970; Price *et al.*, 1970) showed that normal kidney contained both the forms, but only the A form was excreted in the urine. Grebner & Tucker (1973) showed that the urine of normal male individuals contained not only both forms A and B but also another form, M. Earlier workers have shown that form B is excreted only after kidney damage caused by surgical procedure, or in rats after nephrotoxic drug administration (Dance *et al.*, 1969, 1970; Price *et al.*, 1970).

Several forms of hexosaminidase have been isolated from various sources. However, the physiological function is known only for form A. There is no clear relationship between the various forms, but studies on the action of neuraminidase indicate that hexosaminidase A contains more sialic acid residues and that their removal produced forms of similar electrophoretic mobility to the intermediates and to the form B (Robinson & Stirling, 1968; Goldstone *et al.*, 1971; Price & Dance, 1972). Srivastava & Beutler (1972) showed the immunological identity of the A and B form of the enzymes. This observation was confirmed by Carroll & Robinson (1973). They proposed a common subunit of the enzyme for both the forms (Robinson *et al.*, 1973). Carroll & Robinson (1974) isolated a low-molecular-weight protein from human liver, which cross-reacts with hexosaminidases. The glycoprotein nature of the lysosomal hydrolases was shown by Goldstone & Koenig (1970) by periodic acid–Schiff staining after electrophoretic separation. This was further substantiated by Bishayee & Bachhawat (1974*a*,*b*). They have separated and partially purified a few lysosomal acid hydrolases from crude sheep brain lysosomal extracts by concanavalin A–Sepharose affinity chromatography and elution at different pH values in the presence of α-methyl glucoside.

The present paper describes the purification and properties of hexosaminidase A from normal human urine by anion-exchange chromatography followed by affinity chromatography on a concanavalin A–Sepharose column. The composition and the nature

of the carbohydrates present in the enzyme is also reported.

Experimental

Materials

The *p*-nitrophenyl glycosides of β-2-acetamido-2-deoxy-D-glucose, β-2-acetamido-2-deoxy-D-galactose, β-D-glucose, β-D-galactose and naphthol AS-BI 2-acetamido-2-deoxy-β-D-glucose, cyanogen bromide and α-methyl glucoside were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. DEAE-Sephadex A-50 and Sepharose 4B were obtained from Pharmacia Chemicals, Uppsala, Sweden. *p*-Chloromercuribenzoate, GSH, Tris, crystalline bovine serum albumin and Fast Red Violet LB-salt were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. *N*-Ethylmaleimide was the product of Schwarz Bio-Research, Mount Vernon, N.Y., U.S.A., and iodoacetamide was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Concanavalin A was purchased from Biochemical Unit, V.P. Chest Institute, Delhi, India. Glucose, galactose, mannose and fucose were purchased from Pfanstiehl Laboratories, Waukegan, Ill., U.S.A. All other chemicals used were of analytical-reagent grade. All the organic solvents were distilled before use.

Methods

The isolation and identification of carbohydrates was done by the procedure described by Spiro (1966). Protein was determined by using crystalline bovine serum albumin as standard, by the method of Lowry *et al.* (1951).

Polyacrylamide-gel electrophoresis. The purity of the enzyme was evaluated by electrophoresis on 7% (w/v) polyacrylamide gels as described by Davis (1964) at pH 8.3 in Tris–glycine buffer (14.4g of glycine/litre with pH adjusted with solid Tris). Each gel tube contained 100μg of protein. The gels were run at 3mA/tube for 60min at 4°C and the glycoprotein staining was done by the periodic acid–Schiff method (Zacharius *et al.*, 1969). The enzyme staining was done at pH 4.1 in 0.1M-citrate buffer (0.1M-citric acid with pH adjusted with 0.1M-sodium citrate) by the method of Yoshikawa *et al.* (1972).

Enzyme assay. The enzyme was assayed by incubation for 120min at 37°C in a total volume of 0.2ml containing 50μmol of citrate–phosphate buffer, pH 4.5 (1M-citric acid was adjusted with 1M-$Na_2HPO_4,2H_2O$), 200nmol of *p*-nitrophenyl-*N*-acetylglucosamine, 60μg of bovine serum albumin (containing no hexosaminidase activity) and suitable samples of enzyme protein. The reaction was stopped by heating the tube at 100°C for 30s. After cooling the reaction mixture, 0.8ml of 0.4M-glycine–NaOH buffer, pH 10.5 (0.4M-glycine with pH adjusted with 6M-NaOH), was added, mixed and centrifuged for 5min at 2000*g*. The yellow colour formed owing to liberated *p*-nitrophenol was read at 420nm: 1 unit of enzyme is defined as 1nmol of *p*-nitrophenol formed/h. The specific activity is defined as units per mg of protein. For measurement of galactosaminidase activity 49.5nmol of *p*-nitrophenyl *N*-acetylgalactosamine was used as substrate under the standard assay conditions.

Affinity column. The concanavalin A–Sepharose column was prepared by CNBr activation of Sepharose by the procedure of Bishayee & Bachhawat (1974*b*). The concanavalin A content of the column was 6–8mg/ml of Sepharose.

Purification of the enzyme. The pooled urine from male laboratory workers was collected at 28°C and stored for 2h at 4°C. All other operations were carried out at 0–4°C, unless otherwise stated. The column elutions were followed by measuring the enzyme activity with suitable samples of the fractions.

A 2.5-litre batch of urine was dialysed with one change against 4vol. of 0.02M-phosphate buffer, pH 6.0 (0.02M-KH_2PO_4 adjusted with 0.02M-$Na_2HPO_4,2H_2O$), for 18h. The dialysed urine was passed through a DEAE-Sephadex A-50 column (34.5cm×4.5cm diam.) previously equilibrated with the same buffer. The column was washed with 1.5 litres of the same buffer containing 0.15M-NaCl. The enzyme was eluted with 1 litre of the same buffer containing 0.3M-NaCl, and 8ml fractions were collected. The active fractions (nos. 25–65) were pooled and dialysed overnight with one change against 5 litres of

Table 1. *Purification of urinary β-hexosaminidase A*

Details of the purification steps are described in the Experimental section.

	Total volume (ml)	Total protein (mg)	Total activity (units)	Sp. activity (units/mg of protein)	Yield (%)
Urine	10000	8000	261721	32.8	100
Dialysed urine	10000	2587.5	231250	89.4	96
1st DEAE-Sephadex	1140	136.8	116850	854.2	45
2nd DEAE-Sephadex	31	70.99	105300	1483	40
Concanavalin A–Sepharose	70	3.15	63875	20278	25
3rd DEAE-Sephadex	3.1	1.023	32938	32198	13

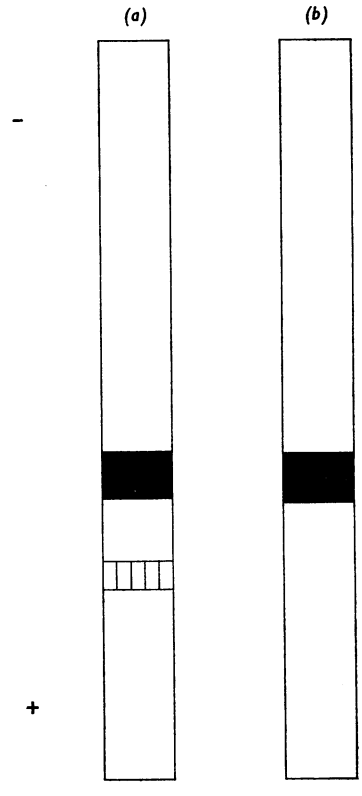

Fig. 1. *Electrophoretic mobility of the enzyme*

(*a*), Glycoprotein staining; (*b*) enzyme staining. Details of electrophoretic conditions and the staining methods are described in the text.

0.02M-phosphate buffer, pH6.0. Eluates from four such 2.5-litre batches of urine were pooled after dialysis and passed through a DEAE-Sephadex column (34cm×2.2cm diam.), which was equilibrated as above. The enzyme was eluted with 0.1M-phosphate buffer, pH6.0 (0.1M-KH_2PO_4 adjusted with 0.1M-$Na_2HPO_4,2H_2O$) containing 0.3M-NaCl, and 8ml fractions were collected. The active fractions (nos. 8–35) were pooled, concentrated by freeze-drying to a smaller volume and dialysed against 10vol. of 0.05M-phosphate buffer, pH7.0 (0.05M-$Na_2HPO_4,2H_2O$ adjusted with 0.05M-KH_2PO_4) for 16h.

The dialysed enzyme solution was passed through a concanavalin A–Sepharose column (10cm×0.8cm diam.) at room temperature. The column was equilibrated with 0.05M-phosphate buffer, pH7.0. The column was washed with 50ml of the same buffer. The active fractions were eluted with the same buffer containing 0.5M-α-methyl glucoside, and 10ml fractions were collected. Fractions 1–6 were pooled. The pooled active fractions from the concanavalin A–Sepharose column were dialysed with two changes against 1 litre of 0.02M-phosphate buffer, pH6.0, for 16h. The dialysed solution was passed through a DEAE-Sephadex A-50 column (9cm×1.8cm diam.), which was equilibrated with 0.02M-phosphate buffer, pH6.0. The column was washed with 100ml of 0.02M-phosphate buffer, pH6.0, containing 0.1M-NaCl and finally eluted with the same buffer containing 0.3M-NaCl; 10ml fractions were collected. Fractions 11–18 were pooled and concentrated to 2.5ml by freeze-drying. The concentrated enzyme preparation was dialysed overnight against 50vol. of 0.02M-phosphate buffer, pH6.0.

Human urinary hexosaminidase A was purified 1000-fold (Table 1) with an overall recovery of 13% by the above procedure. The purified enzyme preparation was completely free from β-glucuronidase, β-galactosidase (Bosmann, 1972) and arylsulphatase A and B activities (Baum *et al.*, 1959). The enzyme could be stored for 2 months at −18°C without any appreciable loss of activity.

The purity of the urinary hexosaminidase A was judged by gel electrophoresis (Fig. 1), which indicated that the preparation was not homogeneous. It showed two glycoprotein bands when stained by the periodic acid–Schiff method but showed only one band when stained for the enzymic activity.

The activity was linear with time for a period of 210min under standard assay conditions. The reaction was linear up to 5μg of purified protein.

Results

pH optimum and kinetic constants

The enzyme had optimum activity at pH4.4–4.5 in citrate–phosphate buffer (Fig. 2) under the standard

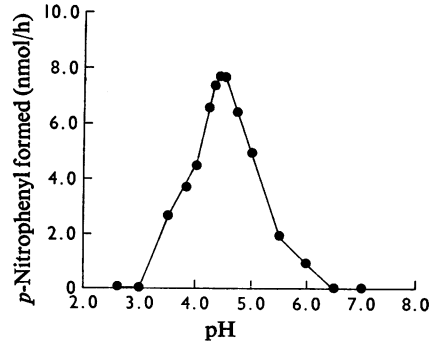

Fig. 2. *pH–activity curve of the enzyme with p-nitrophenyl-N-acetylglucosamine and p-nitrophenyl-N-acetylgalactosamine as substrates*

Details of the experiment are described in the text. The amount of *p*-nitrophenol formed is expressed in nmol.

assay conditions with *p*-nitrophenyl derivatives both of *N*-acetylglucosamine and *N*-acetylgalactosamine. The hexosaminidase A from human spleen (Robinson & Stirling, 1968), liver (Sandhoff & Waessle, 1971; Carroll & Robinson, 1973) and serum (Price & Dance, 1972) had the same pH optimum, whether tested with *p*-nitrophenyl or methylumbelliferyl glycosides.

K_m values obtained from the plot of [S]/*v* against [S] (Dixon & Webb, 1964) for *N*-acetylglucosamine and *N*-acetylgalactosamine derivatives are 0.51 and 0.28 mM respectively (Figs. 3*a* and 3*b*). These values were comparable with K_m values obtained with hexosaminidase from human organs (Robinson & Stirling, 1968; Sandhoff & Waessle, 1971; Carroll & Robinson, 1973), and from jack-bean meal (Li & Li, 1970). The $V_{max.}$ value calculated in the present case is 35.95 μmol/h per mg of protein for the *N*-acetylglucosamine derivative as substrate, and that for the *N*-acetylgalactosamine derivative as substrate is 4.47 μmol/h per mg of protein. $V_{max.}$ values of this order were obtained by Frohwein & Gatt (1967) with calf brain hexosaminidase.

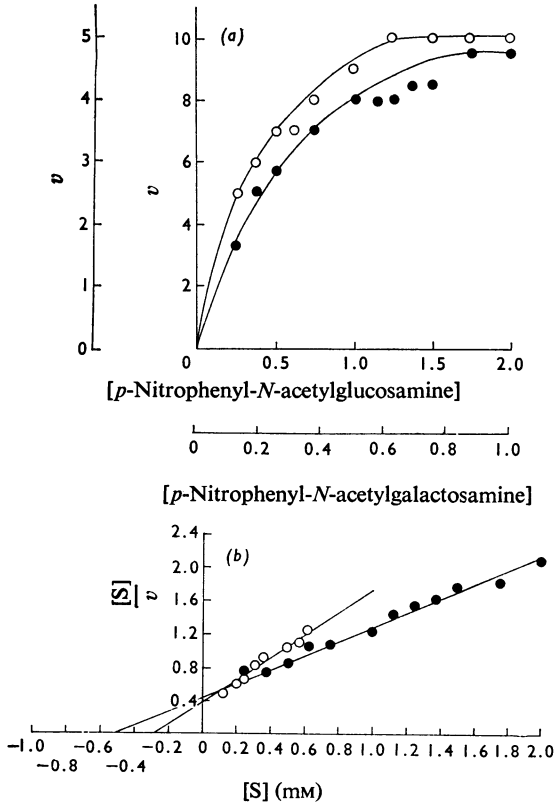

Fig. 3. *Plots of (a) v against [S] and (b) [S]/v against [S]*

Details of the experiments are described in the text. ●, *N*-Acetylglucosamine; ○, *N*-acetylgalactosamine.

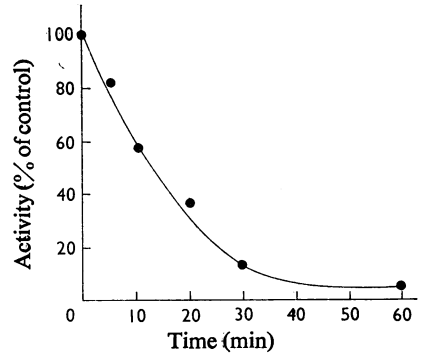

Fig. 4. *Effect of time of incubation at 50°C on enzyme activity*

Details of the experiments are described in the text.

Effect of temperature

The enzyme was preincubated without the substrate at pH 4.5 in citrate–phosphate buffer containing 60 μg of bovine serum albumin for 20 min at temperatures between 0 and 70°C and then assayed at 37°C under standard assay conditions. The enzyme loses its activity above 37°C. Some 50% of the activity was lost in 12 min when incubated at 50°C. The activity of the enzyme after preincubation at 50°C for various periods of time is shown in Fig. 4.

Inhibition studies

The enzyme was preincubated for 15 min at room temperature in the presence of various inhibitors and then assayed under the standard assay conditions. The percentage of activity remaining in the presence of various inhibitors was shown in Table 2. The enzyme was inhibited to the extent of 40% by *p*-chloromercuribenzoate at 0.005 M concentration. But other thiol inhibitors such as iodoacetamide and *N*-ethylmaleimide had less inhibitory effects even at 4 mM concentration. The inhibition exhibited with 0.01 mM-*p*-chloromercuribenzoate could not be reversed even with 0.1 mM concentration of cysteine or GSH. Cysteine or GSH alone did not have any effect. Among the amino sugars *N*-acetylgalactosamine was a more potent inhibitor. Acetate at 17.5 mM concentration inhibited 50% of the activity.

Carbohydrate content

The electrophoresis was carried out as described under 'Methods'. Six gel tubes each containing 100 μg of protein after the third DEAE-Sephadex chromatographic elution were used for the detection of carbohydrates. After the electrophoresis one gel was stained for the enzyme. Another gel was stained for glycoprotein. The gels from the remaining four tubes were

Table 2. *Effect of inhibitors*

Details of the experiments are described in the text.

Additions	Concentration (mM)	Activity (% of control)
None	—	100
GSH	0.1	100
Cysteine	0.1	95
p-Chloromercuribenzoate	0.005	61
	0.01	39
	0.02	23
N-Ethylmaleimide	4	70
Iodoacetamide	4	84
N-Acetylglucosamine	4	73
Glucosamine	4	80
N-Acetylgalactosamine	1	64
	1.5	50
	4	23
Galactosamine	4	83
Acetate	17.5	50
	20	30
Enzyme+*p*-chloromercuribenzoate+GSH*	0.01, 0.1	59
Enzyme+*p*-chloromercuribenzoate+cysteine*	0.01, 0.1	61

* The enzyme was preincubated with the inhibitor, then GSH or cysteine was added and the activity was measured under the standard assay conditions as described in the text.

cut at the region of the enzymic stain. The gels were homogenized with 3ml of 0.1M-phosphate buffer, pH6.0, at room temperature. The homogenate was centrifuged at 17000*g* for 60min. The residue was extracted once more with 3ml of the same buffer. The supernatants were pooled and dialysed for 24h at 4°C against 100vol. of deionized distilled water with two changes.

The non-diffusible material was freeze-dried and the residue was dissolved in 0.2ml of deionized distilled water. A portion was taken for determination of sialic acid by the method of Warren (1959) as modified by Saifer & Gerstenfeld (1962). The remaining solution was made 4M with 11.5M-HCl. The acidic solution was heated in a sealed tube at 100°C for 4h. The hexosamines and neutral sugars were separated and detected by the methods described by Spiro (1966). The following carbohydrates were present in the enzyme molecule: sialic acid, galactose, glucose, mannose and hexosamines. Since the amount was too low, we could not characterize the individual hexosamines.

Discussion

β-Hexosaminidase A was purified from normal male urine by using anion-exchange and concanavalin A–Sepharose affinity chromatography. Although electrophoretically the enzyme was not homogeneous, it showed no other lysosomal acid hydrolase activity except that of acid phosphatase. With the procedure used by us for the purification of the enzyme, we could not detect the presence of the B or M form of the enzyme as reported by Grebner & Tucker (1973). We could not detect the presence of M form of the enzyme in the normal human male urine even by the procedure described by Grebner & Tucker (1973). It was shown by Dance *et al.* (1969, 1970) and Price *et al.* (1970) that normal human urine contained only the A form of β-hexosaminidase.

Dialysed urine before anion-exchange chromatography lost 80% of its β-hexosaminidase activity when kept at −18°C for 24h. However, the enzyme was stable at the same temperature for at least 2 months after DEAE-Sephadex chromatography.

So far as we know this is the first detailed report of the purified urinary enzyme. Brady (1972) reported the purification of the hexosaminidase A from human urine to be used for replacement therapy for patients with Tay–Sachs disease. The glycoprotein nature of the tissue lysosomal acid hydrolases was shown by Goldstone & Koenig (1970) and has been substantiated by Bishayee & Bachhawat (1974*a*,*b*). We have separated the enzyme from other proteins by disc-gel electrophoresis and shown that it is a glycoprotein. The evidence for this has been substantiated not only by affinity chromatography on concanavalin A–Sepharose but also by identifying the constituent carbohydrates present in the enzyme molecule. The presence of glucose and mannose in the eluted enzyme is in accordance with the binding specificity of concanavalin A (Goldstein *et al.*, 1965). The presence of glucose in the purified lysosomal fractions of rat kidney and liver was shown by Goldstone & Koenig (1970). Bishayee & Bachhawat (1974*b*) have shown the presence of glucose in purified lysosomal fractions of sheep brain after elution from a concanavalin A–Sepharose column at different pH values.

The effect of temperature, the electrophoretic mobility and the behaviour on DEAE-Sephadex showed that the enzyme obtained from normal human urine is the A form. The urinary enzyme showed activity towards both the aglycones *N*-acetylglucosamine and *N*-acetylgalactosamine derivatives, as did β-hexosaminidase A from other tissues. Although the K_m for *p*-nitrophenyl-*N*-acetylgalactosamine was lower, the velocity of the reaction was much slower as compared with *p*-nitrophenyl-*N*-acetylglucosamine. Poenaru & Dreyfus (1973) observed that hexosaminidase C was absent from human urine. At the present moment we have no suitable explanation for the complete non-reversal of the inhibition by *p*-chloromercuribenzoate even with a tenfold increase in the concentration of GSH or cysteine. However, the enzyme retained its full activity when *p*-chloromercuribenzo-

ate and cysteine or GSH were added together during preincubation.

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