PURIFICATION OF HUMAN HEXOSAMINIDASES *A* AND *B* AFFINITY CHROMATOGRAPHY

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1. Introduction

The enzyme hexosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamido-deoxy glucohydrolase, EC 3.2.1.30) exists in various human tissues in two major molecular forms designated Hex *A* and Hex *B* [1]. The first one being more acidic and heat labile than the second. These isozymes catalyse the hydrolysis of the glycosidic linkage between *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) and adjacent sugars, and take part in the degradation pathway of several glycosphingolipids including gangliosides [2]. In view of the involvement of these two isozymes and their interrelationships in several lipid-storage diseases, it seems of importance to isolate them in adequate amounts in a pure state so as to enable their chemical characterization. Several procedures were described for the purification of one or both isozymes [3–6]. These include the use of salt precipitation, ion exchange columns, gel filtration and preparative isoelectric focusing. The possibility of using the affinity of the enzyme towards *N*-acetylhexosamines as a tool for purification has also been suggested. Dawson et al. [7] reported partial purification of Hex *A* using immobilized glycopeptide containing the presumable terminal GalNAc. Junowicz and Paris [8] used affinity column of *p*-aminophenyl derivative of GlcNAc bound to Sepharose through an extension arm. Both of these adsorbents are essentially substrate columns, and may therefore suffer 'leakage' of the affinity group from the insoluble matrix due to enzymatic hydrolysis. Hexosaminidase from Jack bean meal have been purified by affinity chromatography on *p*-aminobenzyl-1-thio-*N*-acetylglucosaminide bound to Sepharose [9], by which a 150-fold purification of the enzyme was achieved. Recently, Lotan et al. [10] used an affinity column comprising 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine bound to Sepharose for the purification of wheat germ agglutinin. These authors have observed that the same insoluble matrix may be used for the isolation of hexosaminidase from Jack bean meal as well (personal communication).

We wish to report the use of a similar affinity column for an overall 1200–1500-fold purification of human hexosaminidases.

2. Materials and methods

Ammonium sulfate and cyanogen bromide were obtained from Fluka A. G. (Switzerland). DEAE-cellulose (DE-52) was from Whatman (USA) and Sepharose 4B from Pharmacia (Sweden). All reagents used were analytically pure, or best grade available.

2.1. *Preparation of 2-acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine (CNAG)*

The ligand was prepared and assayed following the procedure described by Lis et al. [11]. The synthesis included the following steps:

(a) Preparation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride by reacting *N*-acetylglucosamine with acetyl chloride.

(b) Conversion of the above chloride derivative to the respective azide by reaction with sodium azide.

(c) Reduction of the azide group to amino by hydrogen in the presence of platinum oxide (Adam's catalyst).

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(d) Binding of the amine to carbobenzoxy-ε-amino-caproic acid in the presence of isobutyl chloroformate and triethylamine.

(e) Deacetylation of the sugar at positions 3,4,6 by sodium methoxide (10% saturated) at pH 8.

(f) Deblocking of the ε-aminocaporyl sugar by hydrogenolysis at atmospheric pressure in the presence of palladium charcoal (10%).

2.2. *Binding of CNAG to Sepharose 4B*

Sepharose 4B was activated by cyanogen bromide at pH 11–12 for 8 min according to Porath et al. [12]. 400 mg of ligand (about 1.4 mmole) were mixed with 40 g activated Sepharose and stirred in the cold for 16 hr. Degree of binding was calculated from the contents of glucosamine and ε-amino caproic acid as determined in Beckman model 120B amino acid analyser following hydrolysis of the washed conjugate. Every gram of Sepharose-derivative contained 0.625 μmole of CNAG.

2.3. *Purification of hexosaminidase A and B*

Human placentae were obtained from the maternity ward of Kaplan Hospital, Rehovot, and homogenized for 3 min in a Waring blender at top speed in chilled 0.01 M phosphate buffer, pH 6. The homogenate was centrifuged at 20 000 *g* in the cold for 30 min. The enzyme was precipitated from the crude extract by the addition of solid ammonium sulfate up to 65% saturation according to the nomogram of Dixon and Webb [13] at pH 7. The precipitate was dissolved in 0.01 M phosphate buffer pH 6 and dialyzed against 6 changes of this buffer for 72 hr. The enzyme solution was loaded on DE-52 column pre-equilibrated with the same buffer. The Hex *B* isozyme was eluted by the equilibrating buffer, whereas Hex *A* was eluted by salt gradient of 0–0.2 M NaCl. Enzyme containing fractions were pooled, dialyzed against 0.01 M phosphate buffer, pH 6, and applied (separately) to the affinity column (Sepharose–CNAG).

2.4. *Physical measurements*

Absorbance was measured in a Zeiss Model PMQ II spectrophotometer. Electrophoresis in 5.3% polyacrylamide gels was run at pH 9.5 according to Hayase et al. [14]. Protein bands were stained with 0.25% Coomassie Brilliant blue.

2.5. *Enzymatic assay*

Enzymatic activity was monitored using fluorescent substrate 4-methyl-umbelliferyl-*N*-acetyl-β-D-glucosamidine [15] at a final concentration of 0.2 mM in 0.04 M citrate buffer pH 4.4, containing 0.1% bovine serum albumin. Fluorescence was recorded in a Turner fluorometer Model 110 using a filter setting for maximal excitation at 365 nm and maximal emission at 450 nm. Enzyme units were calculated according to standard curve of umbelliferone each unit being the amount of enzyme releasing 1 μmole umbelliferone per minute at 37°C.

2.6. *Kinetic studies*

Enzymatic assays of Hex *A* and Hex *B* preparations were performed in various substrate concentrations in the absence or presence of various inhibitor concentrations. The kinetic parameters (K_m and V_{max}) and K_i values were calculated by plotting the results according to Lineweaver and Burk [16] or Dixon [17], respectively.

3. Results and discussion

3.1. *Purification of human placental Hex A and Hex B*

The proportions of Hex *A* and Hex *B* in the crude homogenate and ammonium sulfate precipitate were 62 and 38% (59 and 36 units), respectively, using heat stability as a criterion [18]. Similar values were obtained by measuring the activities present in the Hex *A* and Hex *B* peaks separated on DEAE-cellulose. Specific activity of the enzyme is given in milliunits/O.D. at 280 nm. The materials obtained under the peaks of Hex *A* and Hex *B* from the DEAE-cellulose column (fig. 1). were loaded (separately) on the affinity column (Sepharose–CNAG). The results obtained for the two isozymes are shown in figs. 2 and 3. As depicted, most of the proteins in the solution did not adsorb to the affinity column and were eluted with the starting buffer at pH 6. All the enzymic activity was subsequently eluted in a very minor protein peak by applying 0.01 M phosphate buffer, pH 8.2. As seen in fig. 3 higher purification was achieved by removal of a relatively minor non-enzymatic protein peak at pH 7. The results of the purification procedure are summarized in table 1.

The data of specific activity show that 1220 and

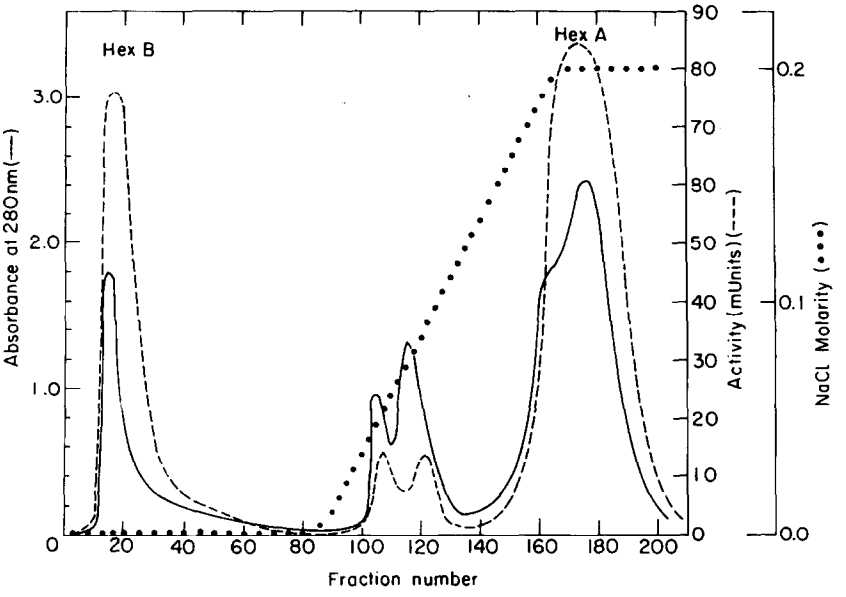

Fig. 1. Elution patterns of hexosaminidase *A* and hexosaminidase *B* from a DEAE-cellulose column. The solid line represents the absorbance, whereas the broken line delineates the enzymatic activity. The salt gradient (NaCl in 0.01 M phosphate buffer, pH 6.0) is given by the dotted curve. The material under the minor peaks (fractions 100–130) was discarded.

1470-fold purification were obtained for Hex *A* and Hex *B* respectively, when compared to the starting material. This relatively high degree of purification was achieved with a small loss of enzymatic activity, most of the loss accounted for by the first step of ammonium sulfate precipitation.

The Hex *A* and Hex *B* peaks obtained from the affinity column were tested for purity by acrylamide gel electrophoresis. The Hex *B* preparation showed the presence of two bands one of which had enzymatic activity. In the Hex *A* preparation there were two protein contaminants accompanying the band with enzymic activity. Efforts are now being made to remove these few contaminants.

Table 1
Purification of Hex *A* and Hex *B*

Isozyme	Preparation	Recovery (percent)	Specific activity (munits/A_{280nm})	Purification
	Crude homogenate	100	59	1
	$(NH_4)_2SO_4$ precipitate	75	96	1.6
Hex *A*	After DEAE-cellulose	68	377	6.4
	After affinity chromatography	67	72 000	1220
	Crude homogenate	100	36	1
	$(NH_4)_2SO_4$ precipitate	77	59	1.6
Hex *B*	After DEAE-cellulose	74	222	6.2
	After affinity chromatography	72	53 000	1470

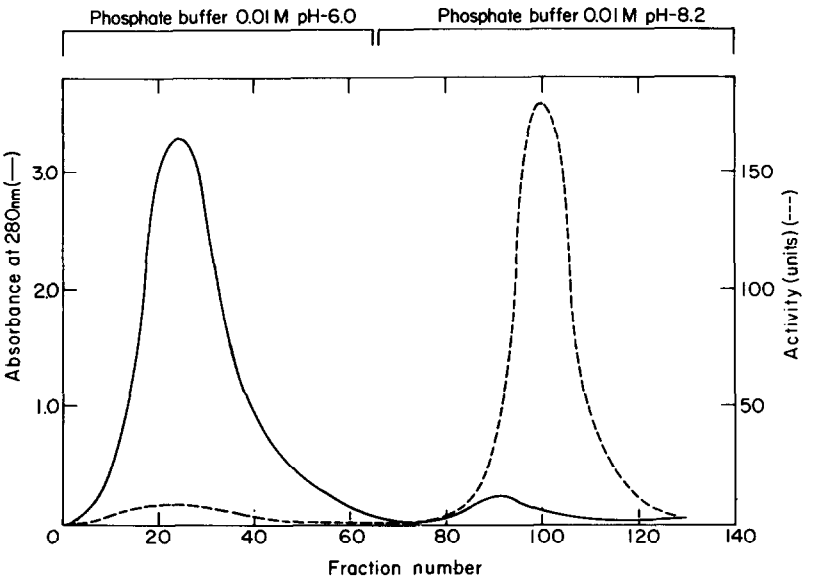

Fig. 2. Elution pattern of hexosaminidase *A* from Sepharose–CNAG column. Solid line – absorbance at 280 nm; broken line – enzymatic activity.

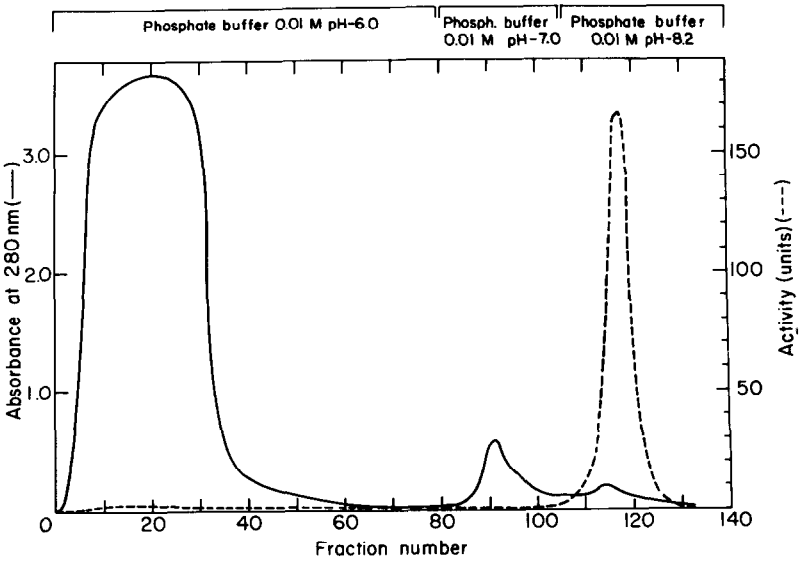

Fig. 3. Elution pattern of hexosaminidase *B* from Sepharose–CNAG column. Solid line – absorbance at 280 nm; broken line – enzymatic activity.

3.2. *Kinetic properties of Hex A and Hex B*

Kinetic studies were carried out with both crude and purified preparations of Hex *A* and Hex *B*, and K_m values for both isozymes were evaluated. The value of K_m did not change with the degree of purity, and was found to be the same for Hex *A* and Hex *B*, as deduced from double reciprocal plot [16]. Fig. 4

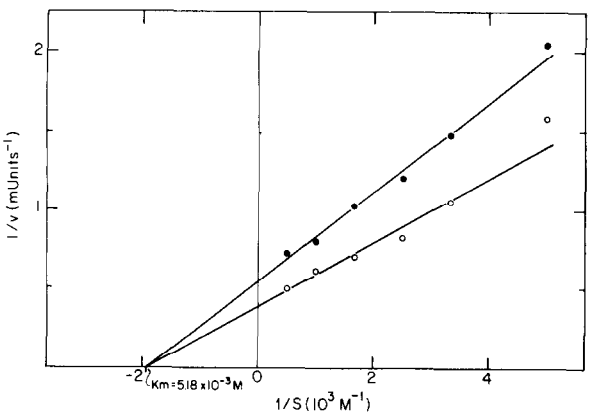

Fig. 4. Double reciprocal plot of the activity of hexosaminidase *A* (•) and hexosaminidase *B* (○). The substrate was 4-methyl umbelliferyl *N*-acetyl-β-D-glucosaminide.

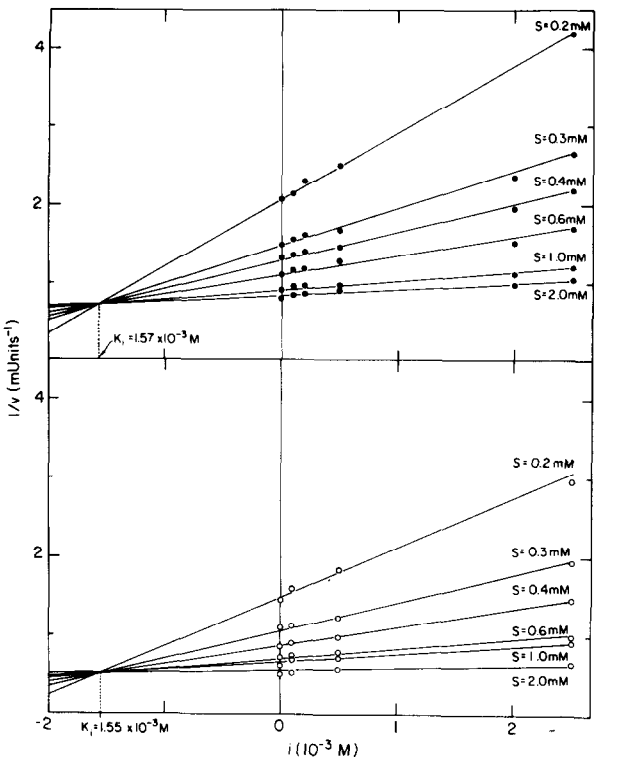

Fig. 5. Kinetics of inhibition of hexosaminidase *A* (upper part) and hexosaminidase *B* (lower part) by CNAG (2-acetamido-*N*-(ε-amino caproyl)-2-deoxy-β-D-glucopyranosylamine). The substrate *(S)* was 4-methyl umbelliferyl *N*-acetyl-β-D-glucosaminide. The pattern illustrates typical competitive inhibition.

demonstrates the results obtained with the purified preparations. Both isozymes were inhibited by the ligand CNAG (which served for preparation of the affinity matrix). The results of the inhibition studies are shown for Hex *A* and Hex *B* in fig. 5. For both isozymes the inhibition by CNAG is purely competitive with an almost identical K_i value (1.57×10^{-3} M for Hex *A* and 1.55×10^{-3} M for Hex *B*).

These data are in accord with the fact that CNAG serves as an appropriate ligand for efficient affinity chromatography for the isolation of hexosaminidases.

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