0273-2289/92/32(1/3)—0171$03.00

Affinity Chromatography of β-*N*-Acetylhexosaminidases on Columns with Mixed Charge and Affinity Functions

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Received July 29, 1991; Accepted November 14, 1991

ABSTRACT

Analysis was made of the nature of interactions between β-*N*-acetylhexosaminidase and affinity chromatography gels made by coupling 2-acetamido-*N*-(6-aminohexanoyl)-2-deoxy-β-*D*-glucopyranosylamine (ANAG) to CNBr-Sepharose columns. This showed that although specific binding of the enzyme to the immobilized ligand was too weak to cause retention, compound affinity in which charge interactions were involved could be exploited for purification of the enzyme. Evidence is given for the specificity of interaction of the enzyme with immobilized ligand and for biospecific desorption of β-*N*-acetylhexosaminidases from ANAG-Sepharose columns. A method was developed for the purification of placental β-*N*-acetylhexosaminidase A in mg amounts starting from crude extracts.

Index Entries: Affinity chromatography; hexosaminidases; human β-*N*-Acetylhexosaminidase A purification.

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INTRODUCTION

β-*N*-Acetylhexosaminidase is a lysosomal enzyme that is widely distributed in nature. In mammalian tissues, it exists in two major forms that can be readily separated from each other by electrophoresis or ion-exchange chromatography. These forms, A (acidic) and *B* (more basic), are composed of αβ- and ββ-subunits, respectively, and have similar substrate specificities in that they can hydrolyze a variety of complex carbohydrates with *N*-acetyl-glucosamine or *N*-acetylgalactosamine in β-linkage at the nonreducing terminal (*1*). β-*N*-Acetylhexosaminidase A is distinguished from B by its ability to hydrolyze ganglioside G_{M2} oligosaccharides from glycosaminoglycans in which subterminal-sugar is negatively charged and from those in which terminal *N*-acetylhexosamine is sulphated at carbon six (*2*). Particular attention has been focused on β-*N*-acetylhexosaminidase A because of its involvement in the autosomal recessive defect Tay-Sachs disease, in which G_{M2} is stored in the lysosomes of nervous tissues.

Several accounts of the purification of human β-*N*-acetylhexosaminidase A have been published, but none of the methods is rapid or convenient (*3–8*). Purifications yielding mg quantities of the enzyme are major undertakings because the enzyme is of low abundance in most mammalian tissues. Although the genes for both α- and β-subunits of the human enzyme have been cloned and their protein sequences deduced (*9,10*), we have a renewed interest in the purification of the enzyme for a variety of reasons that include crystallization and X-ray analysis of the structure, analysis of unusual forms of the enzyme that arise in leukaemic cells (*11*), and obtaining sufficient quantities of the enzyme for functional studies on complex carbohydrates.

Affinity chromatography is often the method of choice for purification of enzymes, and there have been spectacular examples of its application (*12–14*). Affinity methods have proved to be of value as steps in otherwise complex protocols for purification of β-*N*-acetylhexosaminidase (*6*). This affinity method, although demonstratively effective, was of low capacity, used nonspecific elution, and the nature of the interaction between the enzyme and column was not explored. In attempting to repeat this method, we wondered whether a systematic analysis of the interaction might allow us to develop a simpler and higher-capacity method for purification of β-*N*-acetylhexosaminidases.

It is possible, using the method of Graves and Wu, to predict the results of affinity procedures (*15*). This study concerns the binding of β-*N*-acetylhexosaminidase to gels with the ligand ANAG. Assuming that the *Ki* values for binding of immobilized ligand are of the same order as those in free solution (m*M*) and knowing that the concentration of ligand on the gel was also expressed in m*M*, it was apparent that no appreciable retardation of the enzyme on the column as to be expected by affinity in-

teractions alone. In this paper, we explore the interplay between ion-exchange effects caused by the charged isourea linkage, which is formed when ligands with primary amino groups such as ANAG are coupled to CNBr-Sepharose (*16*), and the specificity of binding and elution of β-*N*-acetylhexosaminidase. We demonstrate that β-*N*-acetylhexosaminidase A from human placenta can be purified by a rapid method yielding mg quantities of the enzyme.

MATERIALS AND METHODS

Materials

CNBr-activated Sepharose-4B and CH-Sepharose-4B were obtained from Pharmacia (UK). The 4-Methylumbelliferyl-β-*N*-acetylglucosaminide (4-MUGIcNAc) and naphthyl-AS-BI-β-*N*-acetylglucosaminide were from Koch Light Labs (Colnbrook, Bucks, UK). Monosaccharides, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Coomassie Brilliant Blue, and diazo-5-chloro-o-anisidine were obtained from Sigma Chemical Co. (UK). Ethanolamine was from BDH Ltd., (Poole, Dorset, UK). Placentae were obtained at delivery and liver at post mortem and were kept at −20°C until required.

Methods

Synthesis of Ligands

Glucosylamine was synthesized by the method of Isbell and Frush (1958), and ANAG was synthesized by the method of Koshy et al. (1975) (*17,18*).

Preparation of Tissue Extracts

Extracts of tissues were made by homogenizing 400 g in 1 L of 10m*M* sodium phosphate buffer, pH 6.8, and centrifuging at 10000*g* for 15 min at 4°C to remove debris. The clear supernatant was brought to 30% saturating by adding solid $(NH_4)_2SO_4$ and stirred at 4°C for 4 h. The precipitate was removed by centrifugation as before, and the supernatant was brought to 60% saturation with $(NH_4)_2SO_4$ and stirred gently at 4°C overnight. The resulting precipitate was resuspended in 10m*M* sodium phosphate buffer, pH 6.8, and dialysed against the same buffer.

Synthesis of Chromatography Gels

Structures of the three chromatography matrices (I, II, and III) are shown in Fig. 1. They were synthesized as follows: For gel I, CH-Sepharose was allowed to hydrate in 0.5*M* NaCl for 15 min, transferred to a Pyrex G4 sintered filter, washed with the salt solution (200 mL/g of gel), and re-

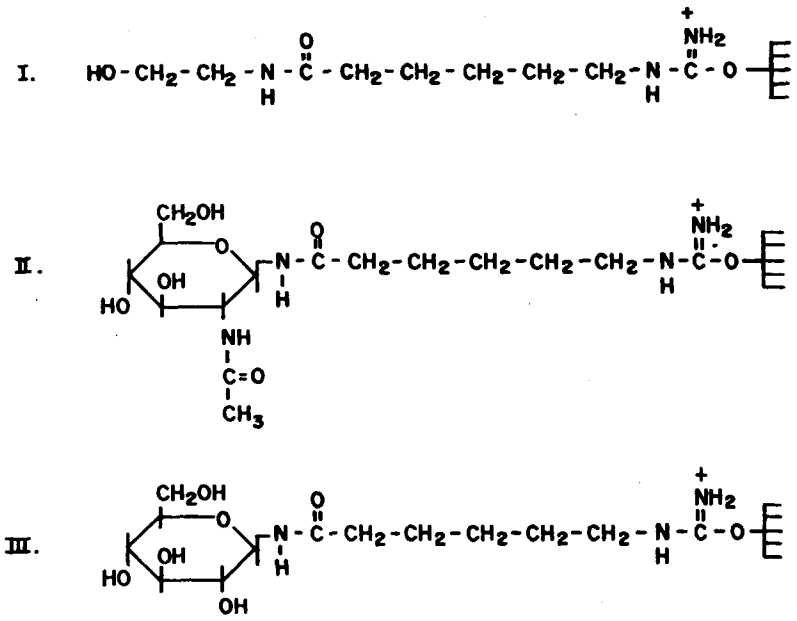

Fig. 1. Structures of immobilized ligands used for purification of β-*N*-acetylhexosaminidase (II) and investigation of the specificity of binding (I and III).

suspended in a minimum quantity of water adjusted to pH 4.5 by the addition of HCl. Ethanolamine (1 mmol/mL of gel) and EDC (50 mmol/mL of gel) were dissolved in a minimum vol of dilute HCl, pH 4.5. The ligand solution was added to the gel first, and the EDC solution was added gradually with gentle stirring. Mixing was continued for the first 90 min of the reaction, and the pH was kept within the 4.5–6.0 range by the addition of HCl. Gentle agitation of the suspension was continued for 16 h at 4°C, and the gel was washed on a glass sinter with dilute HCl, pH 4.5 (200 mL/g of gel) and stored at 4°C. Gel II was prepared by mixing ANAG solution (12 μmol/mL in 0.1*M*-$NaHCO_3$, pH 8.3 containing 0.5*M* NaCl) with CNBr-activated Sepharose-4B, washed according to the manufacturer's instructions in the proportions of 2 mL of ANAG solution for each mL of gel. Gel III was prepared in the same way as gel I except that glucosylamine (200 μmol/mL of gel) was used instead of ethanolamine, with a 50-fold molar excess of EDC. Unreacted carboxyl groups on the CH-Sepharose were blocked by coupling them to ethanolamine under the conditions given for gel I.

β-N-Acetylhexosaminidasae Assay and Detection in Polyacrylamide Gels

β-*N*-Acetylhexosaminidase was assayed by the method of Braidman et al. (1974) using the fluorigenic substrate 4-MUGlcNAc (*19*). One U of enzyme activity is the amount of enzyme that hydrolyzes 1 μmol/min of substrate. β-*N*-Acetylhexosaminidase was detected in gels by incubating

them in 0.1*M*-citric acid/Na_2HPO_4 buffer, pH 4.5, for 20 min at room temperature and then transferring them to a solution of naphthyl-AS-BI-β-*N*-ace-tylglucosaminide (0.6 mg/mL) and diazo-5-chloro-o-anisidine (2 mg/mL) in the same buffer. Incubation was continued until the bands of enzyme activity reached the desired intensity; the gels were then stained with a 0.02% (w/v) solution of Coomassie Brilliant Blue in 10% (v/v) acetic acid.

Polyacrylamide Gel Electrophoresis and Immunodetection

Conditions for electrophoresis in polyacrylamide gradient gels and in sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and methods for Western blotting and immunodetection are those described by Dewji and colleagues (1986) (*8*).

RESULTS

Behavior of β-*N*-Acetylhexosaminidases on Gel I

In gel I, the ligand-coupled CH-Sepharose was hydroxylamine. Application of liver extract (2.5 mL) resulted in almost complete retention of β-*N*-acetylhexosaminidase activity (2.5 U), and this could be recovered by application of a gradient of NaCl (Fig. 2). There was partial separation of the B (the first peak) and A forms under these conditions. Incorporation of 60 m*M* *N*-acetylglucosamine in the eluting buffer had no effect on the displacement of the enzyme from the column.

Behavior of β-*N*-Acetylhexosaminidase on ANAG-Sepharose (Gel II): The Effect of NaCl Concentration on Binding of the Enzyme

Liver extract (1 mL) containing β-*N*-acetylhexosaminidase (0.7 U) was applied to columns containing 5 mL of gel under varying conditions of NaCl concentration. Retained enzyme at each salt concentration was eluted with 2*M* NaCl and assayed for activity. Results show a decreasing capacity of the column for β-*N*-acetylhexosaminidase with increasing salt concentration (Fig. 3).

Separation and Purification of β-*N*-Acetylhexosaminidases A and B on ANAG-Sepharose Columns

By manipulating the concentration of NaCl in the buffers used to load and elute β-*N*-acetylhexosaminidase from ANAG-Sepharose, it was pos-

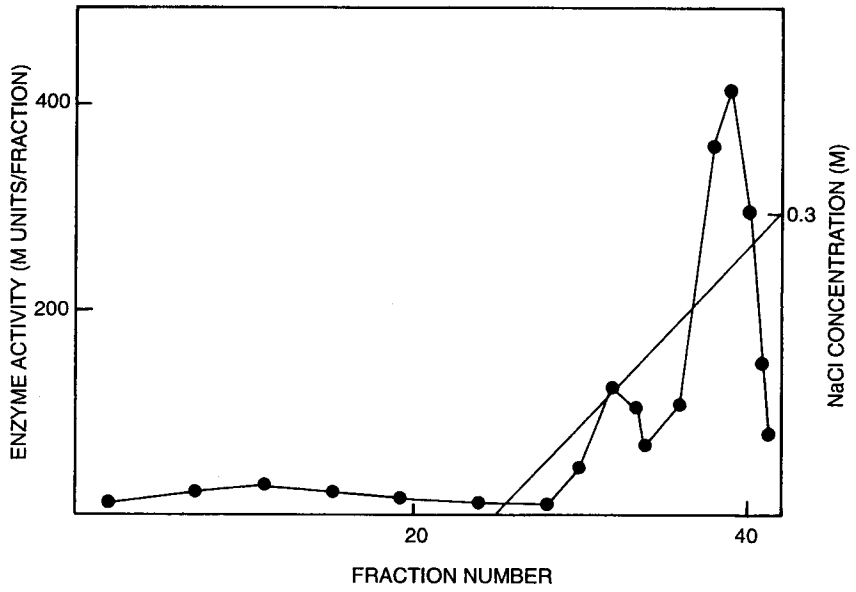

Fig. 2. Nonspecific absorption and elution of human liver β-*N*-acetylhexosaminidase on a gel containing ethanolamine coupled to CH-Sepharose-4B (gel I). Enzyme (2.5 U) was applied (0.5 mL/min) to a column containing 5 mL of the gel in 10m*M* sodium phosphate buffer, pH 6.8. A gradient of NaCl (0–0.3*M*) in buffer was applied in fraction 26.

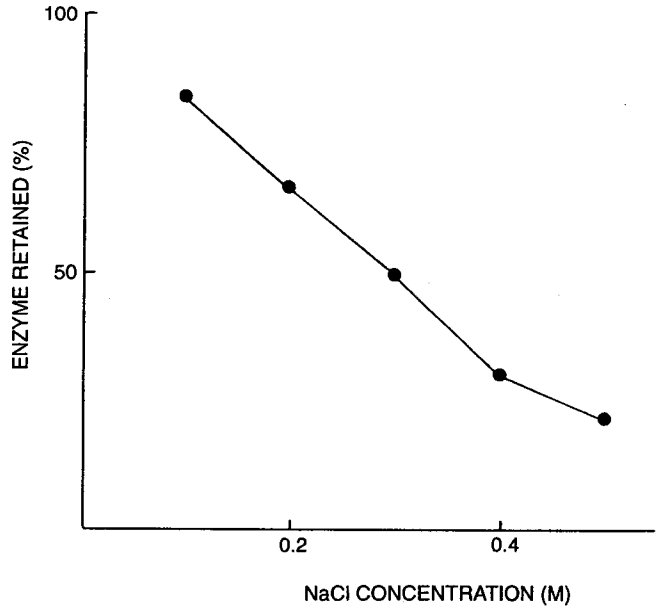

Fig. 3. Relationship between NaCl concentration and the amount of human liver β-*N*-acetylhexosaminidase bound by ANAG coupled to CNBr-Sepharose-4B (ANAG-Sepharose, gel II). Enzyme (0.7 U) was applied to columns containing 5 mL of the gel equilibrated with 10m*M* sodium phosphate buffer, pH 6.8, containing various concentrations of NaCl (0–0.5*M*). Enzyme retained by the column was eluted in each case with 2*M* NaCl and assayed for β-*N*-acetylhexosaminidase activity.

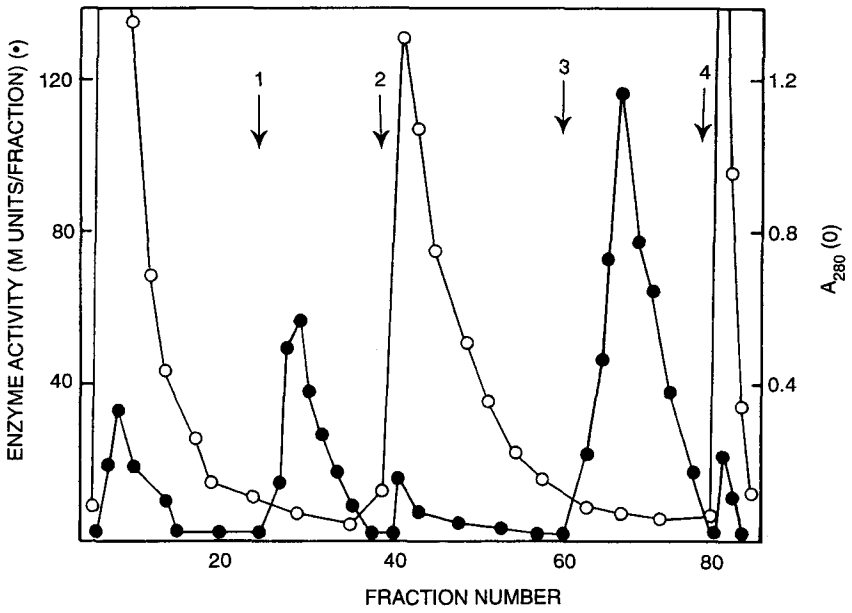

Fig. 4. Affinity chromatography of human liver β-*N*-acetylhexosaminidase on ANAG-Sepharose (gel II). Enzyme (2 U) was applied (0.5 mL/min) to a column containing 5 mL of gel equilibrated with 10m*M* sodium phosphate buffer, pH 6.8, containing 30m*M* NaCl. The column was eluted with 10m*M* sodium phosphate buffer, pH 6.8, containing (*1*) 30 m*M* NaCl and 60m*M* GlcNAc, (2) 70m*M* NaCl, (3) 70m*M* NaCl and 60m*M* GlcNAc, and (4) 2*M* NaCl.

sible to select conditions in which a high proportion of the enzyme was retained and to achieve selective desorption of the A and B forms from the same column with GlcNAc (Fig. 4). In preliminary experiments, a concentration of salt just lower than that required to elute the particular form was selected, and inclusion of GlcNAc (*Ki* 12 m*M*) under these conditions resulted in desorbtion of the enzyme. Form B was eluted at the lower salt concentration and A at the higher. Their identities were confirmed by electrophoresis (data not shown).

Specificity of Elution of β-*N*-Acetylhexosaminidase from ANAG-Sepharose by Monosaccharides

Analysis of the specificity of desorption of β-*N*-acetylhexosaminidase from ANAG-Sepharose was performed under conditions similar to those described in the legend of Fig. 4, except that the test sugar was used first in the eluting buffer, followed in each case by GlcNAc. Three of the results are given in Fig. 5, which shows that sugars that inhibit the enzyme in solution GalNAc (*Ki* 0.6 m*M*) (Fig. 5a) and mannose (*Ki* 30 m*M*) (Fig. 5b) give complete desorption of the isoenzymes at the appropriate salt concentrations. However, *L*-fucose, which does not inhibit β-*N*-acetylhexosa-

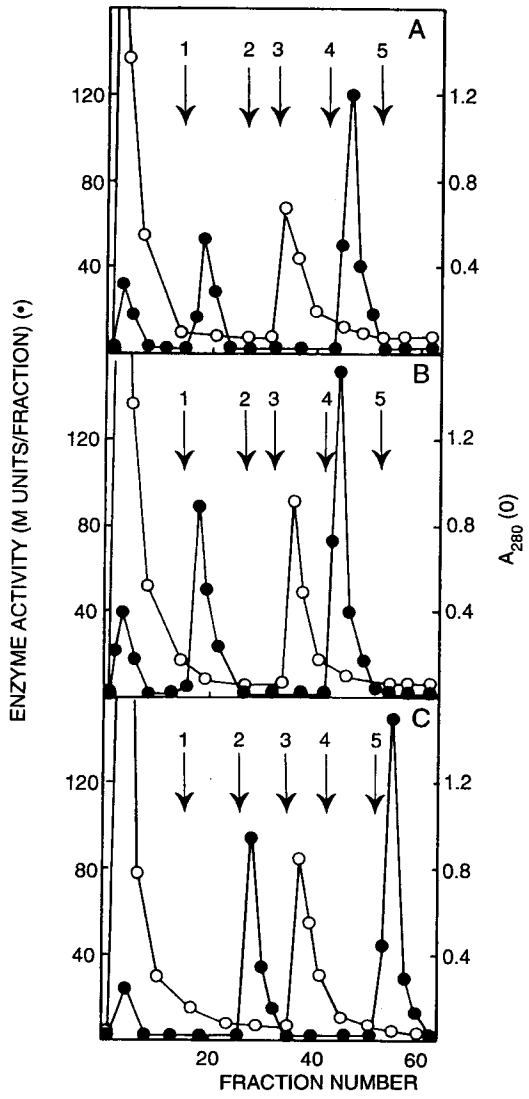

Fig. 5. Effect of (**A**) *N*-acetyl-*D*-galactosamine, (**B**), *D*-mannose, and (**C**) *L*-fucose on the displacement of human liver β-*N*-acetylhexosaminidases bound to ANAG-Sepharose. In each case, enzyme (1.0 U) was applied to a column containing 3 mL of the gel equilibrated with 10m*M* sodium phosphate buffer, pH 6.8, containing 30m*M* NaCl. Columns were eluted with (**A**) (*1*) 60m*M* GalNAc and 30m*M* NaCl, (*2*) 60m*M* GlcNAc and 30m*M* NaCl, (*3*) 70m*M* NaCl, (*4*) 60m*M* GlcNAc and 70m*M* NaCl, and (*5*) 60m*M* GlcNAc and 70m*M* NaCl; (**B**) (*1*) 60m*M* Man and 30m*M* NaCl, (*2*) 60m*M* GlcNAc and 30m*M* NaCl, (*3*) 70m*M* NaCl, (*4*) 60m*M* Man and 70m*M* NaCl, and (*5*) 60m*M* GlcNAc and 70m*M* NaCl; and (**C**) (*1*) 60m*M* fuc and 30m*M* NaCl, (*2*) 60m*M* GlcNAc and 30m*M* NaCl, (*3*) 70m*M* NaCl, (*4*) 60m*M* fuc and 70m*M* NaCl, and (*5*) 60m*M* GlcNAc and 70m*M* NaCl.

minidase, is ineffective (Fig. 5c). Identical results were obtained for *D*-arabinose, *D*-fructose, *D*-glucose, *D*-galactose, *D*-tagatose, and *D*-sorb se; none of which is an inhibitor of the enzyme.

Effect of Substituting 2-Acetamido-2-Deoxyglucopyranosylamine (Gel II) With Glucopyranosylamine (Gel III)

In order to assess the importance of ligand specificity in determining the behavior of β-*N*-acetylhexosaminidase on affinity gels, a liver extract containing 1.7 U of enzyme was applied to gel III in 10 m*M* sodium phosphate buffer, pH 6.8. Most of the enzyme was retained (80%), and 90% could be recovered by elution with the same buffer containing 70 m*M* NaCl. Under the conditions shown in the legend of Fig. 4, there was no specific elution of β-*N*-acetylhexosaminidase by GIcNAc. Bound enzyme was eluted slowly in 10m*M* sodium phosphate buffer, pH 6.8, containing 50 m*M* NaCl, but this elution was not accelerated by inclusion of 60 m*M* GIcNAc. β-*N*-acetylhexosaminidase was not inhibited by 60m*M* glucosylamine.

Purification of Human Placental β-*N*-Acetylhexosaminidases A

A placental extract containing 675 U of β-*N*-acetylhexosaminidase (380 mL) was applied to an ANAG-Sepharose column (40 mL) in 10m*M* sodium phosphate buffer, pH 6.8, containing 0.1 M-NaCl (Fig. 6). There was a gradual increase in the percentage of the enzyme unbound by the column, but application of the extract was stopped well before the capacity of the column was reached. Two main peaks of activity were recovered with specific activities of 44 U/mg of protein (B) and 88 U/mg (A) (Table 1).

On nondenaturing polyacrylamide gel electrophoresis (Fig. 7), the A form gave a single sharp band when stained for enzyme activity, and post-stained for protein. The B form was not completely pure and was found to be contaminated with IgG (data not shown). Electrophoresis of the A form on SDS-PAGE under denaturing conditions (Fig. 8) showed bands at 53000 and 29000, the expected sizes of the α- and β-subunits, respectively.

DISCUSSION

Coupling of ligands to gel matrices activated with CNBr has long been considered disadvantageous because of the introduction of a charged isourea linkage with a *pKa* value of about 9.5 (*14,16,20*). Such affinity gels are at the best-mixed function matrices having specific and ionic functions,

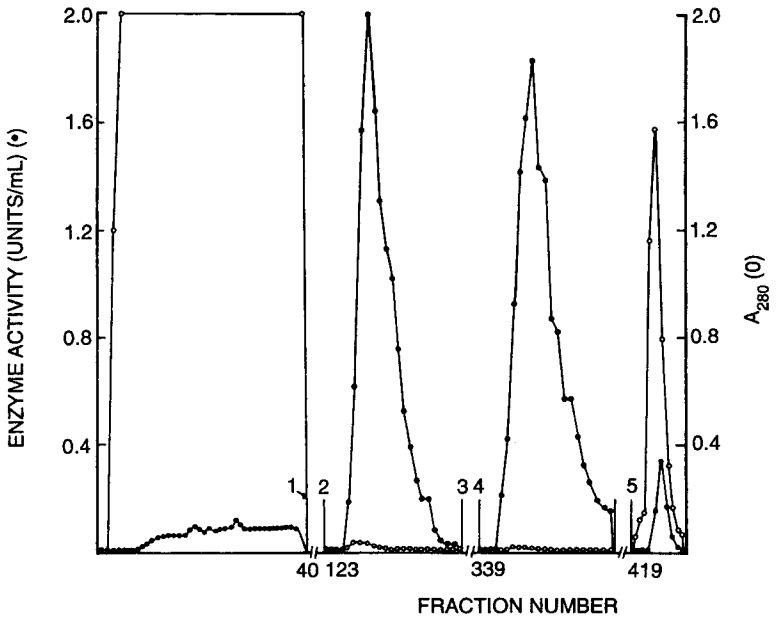

Fig. 6. Preparative affinity chromatography of human placental β-*N*-acetylhexosaminidases on ANAG-Sepharose. Enzyme (675 U in 380 mL of extract) was applied to a 40 mL column of ANAG-Sepharose equilibrated with 10m*M* sodium phosphate buffer, pH 6.8, containing 0.1*M* NaCl. Fractions (10 mL) were collected at a flow rate of 40 mL/h. The column was eluted with 10m*M* sodium phosphate buffer, pH 6.8, containing (*1*) 0.1*M* NaCl (495 mL) then 30m*M* NaCl (345 mL), (*2*) 30m*M* NaCl and 60m*M* GlcNAc (240 mL), (*3*) 30m*M* NaCl to elute the sugar of the previous solution (80 mL) then 0.1*M* NaCl (1.6 L) and 70m*M* NaCl (240 mL), (*4*) 70m*M* NaCl and 60m*M* GlcNAc (0.8 L), and (*5*) 1*M* NaCl (100 mL).

and the latter may be responsible for nonspecific binding of contaminating proteins to the matrix (*14*). Where the affinity between an enzyme and an immobilized ligand is weak, the anion-exchange properties of these gels may become dominant. When an aliphatic amine is coupled to a CNBr-activated matrix, the charge may be delocalized and distributed over the surface of the gel (*14*).

In the affinity purification of β-*N*-acetylhexosaminidase that we have described here, the specific ligand (*N*-acetylglucosamine) was coupled through an amide linkage to 6-aminohexanoic acid (Fig. 1II) that was in turn coupled to CNBr-Sepharose. Thus, there may be affinity, charge, and hydrophobic components in the interaction between the enzyme and the ANAG column. The effect of increasing NaCl concentration was to decrease the binding of the enzyme to the column. This may indicate the importance of ion-exchange effects in the interaction. Elution of the B form of β-*N*-acetylhexosaminidase at a lower concentration of salt than that of A is also consistent with the charge effect being dominant.

Table 1
Purification of β-*N*-Acetylhexosaminidases on ANAG-Sepharose

Preparation	Vol (mL)	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Buffer extract	1162.0	1042	25.3×10^3	0.04		100
30–60% sat. $(NH_4)_2SO_4$	380.0	675	4.9×10^3	0.14	3.5	65
ANAG-Sepharose A peak	1.4[a]	210	2.4	88.00	2200[b]	31
ANAG-Sepharose B peak	1.6[a]	144	3.3	44.00	1100[b]	21

[a] After concentration.
[b] Final purification factors were calculated on the basis of total β-*N*-acetylhexosaminidase activity of the buffer extract.

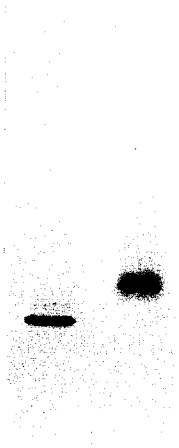

Fig. 7. Polyacrylamide gradient gel electrophoresis of β-*N*-acetylhexosaminidases. Samples containing 10 μg of protein were applied to a 3–26% gradient polyacrylamide gel and electrophoresed for 46 h. The gel was then stained for enzyme activity with naphthyl-AS-BI-β-*N*-acetylglucosaminide as substrate and diazo-5-chloro-o-anisidine as the coupling dye and poststained for protein with Coomassie blue. Lanes A and B contained β-*N*-acetylhexosaminidases A and B, respectively.

Our observation that β-*N*-acetylhexosaminidase could be desorbed from its interaction with ANAG-Sepharose with a competitive inhibitor, GlcNAc, encouraged us to find optimal conditions in which the salt concentration was adjusted to a value just below that required to elute a particular form of the enzyme before applying biospecific desorption with GlcNAc. Thus, the behaviour of β-*N*-acetylhexosaminidase on ANAG-Sepharose columns falls into the category of compound affinity effects described by O'Carra and co-workers (1974) in which a weak specific interaction is augmented by a nonspecific effect (*21*).

Desorption of β-*N*-acetylhexosaminidases A and B from ANAG-Sepharose was specific in the sense that they were desorbed only by sugars that inhibited the enzymes in free solution. We can see no stereochemical similarity between *D*-mannose and *N*-acetyl-*D*-glucosamine that would account for its inhibitory effect on β-*N*-acetylhexosaminidase, but it has been noted previously with the enzymes from bovine epididymis and porcine placenta (*22*). This inhibitory effect of *D*-mannose might indicate the presence of a more complex binding site than one that accommodates a terminal *N*-acetylhexosamine alone.

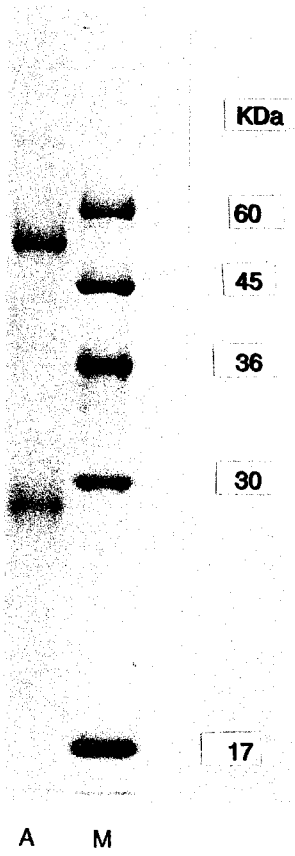

Fig. 8. Electrophoresis of β-*N*-acetylhexosaminidase A in a polyacrylamide gel containing sodium dodecyl sulphate. A sample of β-*N*-acetylhexosaminidase A was denatured in sample buffer containing 50m*M* dithiothreitol and electrophoresed on a 10% polyacrylamide gel containing sodium dodecyl sulphate. Polypeptides were stained with Coomassie blue. Lane A, β-*N*-acetylhexosaminidase A; lane M, mol wt markers.

Desorption of an enzyme from an affinity chromatography column with a specific free ligand does not in itself constitute evidence of a specific interaction between the enzyme and the immobilized ligand. Scopes (1977) demonstrated that a variety of enzymes of intermediary metabolism could be eluted from columns of CM-cellulose by sequential elution of the column with their substrates (23). We believe, however, that the interaction between β-*N*-acetylhexosaminidase and ANAG-Sepharose does involve a specific component, for two reasons: Biospecific elution of the type exploited by Scopes (1977) involved binding of a negatively charged substrate with a concomitant change in the net charge of the enzyme on formation of the enzyme-substrate complex. In the system we have described here, none of the competitive inhibitors was charged or,

for that matter, had hydrophobic character. Furthermore, specific elution was impossible from gels I and III, in which the general character of the immobilized ligand was retained and the GlcNAc was replaced with ethanolamine and glucose, respectively. Whether the presence of the hydrophobic spacer contributes to the interaction will only be possible to assess by comparison with gels in which the spacer is given some hydrophilic character.

Our objective in analysing the behaviour of β-*N*-acetylhexosaminidase on ANAG-Sepharose was to devise a high-capacity, simple method for purification of the A form. Scaling up purification of the liver enzyme was limited by the capacity of the columns for nonspecific binding, although this was ameliorated to some extent by increasing the concentration of NaCl in the extract to 0.1*M*. The capacity of columns for placental extracts was greater, and in the example we have given here, 2.4 mg of β-*N*-acetylhexosaminidase A were purified on a 40mL column. This was below the functional capacity of the column. Purified A gave a single band on nondenaturing polyacrylamide gradient gel electrophoresis that stained for enzyme and protein (Fig. 7). SDS-PAGE under denaturing conditions gave the expected sizes of the proteolytically processed α- and β-subunits (Fig. 8).

Purification of β-*N*-acetylhexosaminidase B, although remarkable in comparison with conventional purification methods, did not yield pure enzyme under these conditions. Smaller-scale purifications in which less extract was applied to the column yielded pure B with a specific activity of 134 U/mg of protein under assay conditions. The reason for this difference is not apparent, but it is possible that there is a β-*N*-acetylhexosaminidase–contaminating protein interaction that becomes more evident when other nonspecific binding sites on the column are saturated. We have previously used the ANAG-Sepharose affinity method, without proper characterization, in a complex protocol for purification of β-*N*-acetylhexosaminidase I from liver (*8*) and in the purification of the enzyme from boar epididymis (*24*). In both instances, the enzyme was already of high specific activity before application to ANAG-Sepharose. The method we have described here for purification of β-*N*-acetylhexosaminidase A from a crude $(NH_4)_2SO_4$ fraction has enabled us to purify a sufficient amount of the enzyme for structural studies. We have preliminary evidence that α-*L*-fucosidase and β-*D*-glucosidase may be purified by a similar strategy using appropriate ligands.

ACKNOWLEDGMENTS

Luana C. B. B. Coelho is grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brasil and the Universidade Federal de Pernambuco for financial support. Winston Hutchinson held an SERC (Case) Studentship.

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