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AN AFFINITY ADSORBENT FOR *N*-ACETYL-β-D-HEXOSAMINDIASE A

EUGENE E. GREBNER[a] and INDU PARIKH[b]

[a]*Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pa. 19141 and* [b]*Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205 (U.S.A.)*

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SUMMARY

p-Aminophenyl-*N*-acetyl-β-D-thioglucosamine was coupled to succinylated diaminodipropylamino-agarose to produce an affinity adsorbent which selectively adsorbed urinary *N*-acetyl-β-D-hexosaminidase A (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30). Chromatography of unfractionated urinary proteins on this adsorbent yielded partially purified *N*-acetyl-β-D-hexosaminidase A from which had been removed about 90% of the contaminating protein, including the B isozyme.

INTRODUCTION

Deficiency of *N*-acetyl-β-D-hexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) is the cause of two heritable sphingolipidoses: Tay–Sachs disease (deficiency of the A isozyme of *N*-acetyl-β-D-hexosaminidase) and Sandhoff disease (deficiency of both A and B isozymes) [1–3]. Human A and B isozymes have been purified for studies related to these deficiencies [4–7] but conventional purification procedures are tedious and yields are often low. To facilitate purification of these enzymes we have developed an adsorbent (with *p*-aminophenyl-*N*-acetyl-β-D-thioglucosamine as ligand) of potential usefulness for the affinity chromatography of *N*-acetyl-β-D-hexosaminidase A.

METHODS

Preparation of ligand and affinity adsorbent

Synthesis of the ligand (*p*-aminophenyl-*N*-acetyl-β-D-thioglucosamine) was based on a modification of a published method for the synthesis of the corresponding oxygen analog [8]. Acetochloroglucosamine (10.0 g, 27.4 mmoles), prepared as in ref. 8, and 5.7 g (41.0 mmoles) *p*-nitrothiophenol were stirred in 100 ml acetone for 10 min followed by the gradual addition of 41 ml 1 M NaOH. After standing 15 h at 4 °C the microcrystalline *p*-nitrophenyltetraacetyl-β-D-thioglucosamine was filtered and

Abbreviation: 4-MU-GlcNAc, 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide.

washed with cold 0.05 M NaOH until washings were colorless. The yield was 4.2 g as a slightly yellow product, m.p. 185–187 °C, which was used for the next step without further purification. The mass spectrum of this compound showed an M—154 peak at *m/e* 330 confirming the molecular weight of 484 for the derivative. Cleavage of acetal bonds characteristic of glycosides was detected in the mass spectrum.

100 mg *p*-nitrophenyl-tetraacetyl-β-D-thioglucosamine with 20 mg Pd/C (10%) in 5 ml methanol was reduced in a Parr apparatus with H_2 gas at 30 lb/inch^2 for 10 h. The reaction mixture was centrifuged and the pellet washed twice with 5 ml methanol containing 0.1 ml 1.0 M HCl. The combined methanol extracts were dried in vacuo, redissolved in 5 ml methanol and added to a 1:1 suspension in dioxane of 25 ml succinylated diaminodipropylamino-agarose [9] previously washed with 200 ml dioxane. Dicyclohexylcarbodiimide (100 mg) was added and the suspension gently shaken for 6 h at 24 °C. The substituted agarose was filtered and washed in a coarse disc sintered glass funnel with 250 ml dioxane (1 h) followed with 250 ml methanol (1 h). The agarose was resuspended in 25 ml of methanol–dioxane (2:1, v/v) and 1.0 ml of 0.01 M methanolic sodium methoxide was added followed with gentle shaking in a polyethylene bottle for 2 h at 24 °C. The agarose-diaminodipropylamino-succinyl-*p*-aminophenyl-*N*-acetyl-β-D-thioglucosamine was filtered and washed with 250 ml methanol (1 h), 250 ml dioxane (1 h), and 500 ml water. The substitution of *N*-acetylglucosamine on agarose, as judged by the recovery of the unreacted ligand, was between 5 and 7 μmoles per ml of agarose adsorbent. Before use the resin was equilibrated with the same buffer in which the protein was to be applied. After chromatography the column was stripped with 6 M urea in 1 M NaH_2PO_4 followed with a buffer wash. The resin could be used repeatedly.

Partial purification and separation of N-acetyl-β-D-hexosaminidases A and B

The protein fraction which precipitated between 20 and 60% $(NH_4)_2SO_4$ concentration was prepared from freshly voided human urine and dialyzed against 0.01 M phosphate buffer, pH 6.1, containing 0.4 M NaCl, followed by chromatography on a Sephadex G-200 column using the same buffer. Enzymatically active fractions from the Sephadex column were pooled and concentrated by precipitation with 80% $(NH_4)_2SO_4$. The precipitate was taken up in and dialyzed against 0.01 M phosphate buffer, pH 6.1, and the retentate was chromatographed on a DEAE-cellulose column to separate the A and B isozymes as previously described [10]. The A and B isozymes obtained in this manner were concentrated by $(NH_4)_2SO_4$ precipitation, dialyzed against 0.06 M phosphate buffer, pH 6.1, and the sample applied to the affinity column. Columns were operated at 4 °C with a flow rate of about 15 ml/h.

Protein was determined by the method of Sutherland et al. [11]. Polyacrylamide gel electrophoresis was carried out by the method of Gabriel [12] (gel formulation 6), and stained for protein by the method of Fairbanks et al. [13].

RESULTS AND DISCUSSION

When partially purified *N*-acetyl-β-D-hexosaminidase A was applied to the affinity column in 0.06 M phosphate buffer, pH 6.1, all the enzymatic activity was adsorbed while most of the protein passed through with the void volume. Enzyme activity was subsequently eluted with 0.2 M borate buffer, pH 8.0, (Fig. 1). 1 ml of

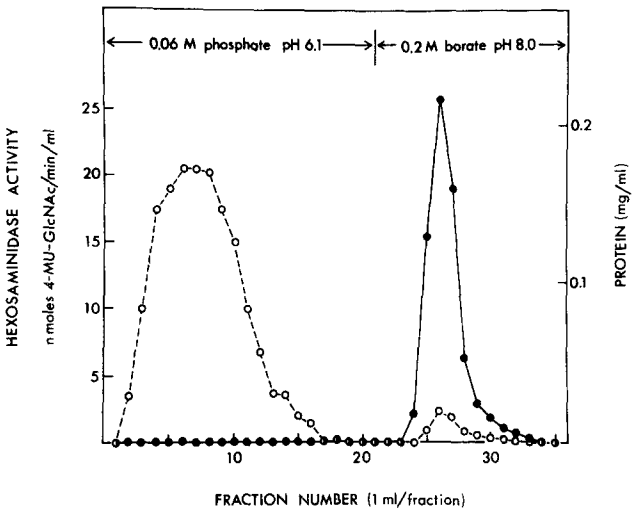

Fig. 1. Affinity chromatography of partially purified *N*-acetyl-β-D-hexosaminidase A. 9.8 ml of a partially purified *N*-acetyl-β-D-hexosaminidase A preparation containing approx. 1.5 mg protein was applied to a small affinity column (1.2 cm × 2.5 cm) in 0.06 M phosphate buffer, pH 6.1. After washing with this buffer the enzyme was eluted with 0.2 M borate buffer, pH 8.0. Reaction mixtures for enzyme assays consisted of 100 μl 0.1 M citrate–phosphate buffer, pH 4.5, 20-μl sample, and 100 μl 1.0 mM 4-MU-GlcNAc in the same buffer. After incubation at 37 °C for 10 min, 4.75 ml of 0.15 M glycine–carbonate buffer, pH 10.0, was added and fluorescence measured with a Farrand fluorometer using 7-60 (primary) and 47B+2A (secondary) filters. Solid line represents *N*-acetyl-β-D-hexosaminidase activity, dotted line represents protein.

affinity adsorbent was able to bind approx. 0.21 unit (1 μmole substrate cleaved per min) of enzyme. Application of the material at buffer concentrations lower than 0.06 M permitted non-specific binding of some of the protein (at 0.01 M all the protein was bound). Higher buffer concentrations resulted in slight leakage of the enzyme. Elution of the enzyme could also be accomplished with 0.2 M phosphate buffer, pH 6.1, but the activity peak showed considerable trailing. The enzyme was not eluted with any of the following: 0.1 % Triton X-100, 0.5 M sodium acetate buffer, pH 4.5, 0.5 M *N*-acetylglucosamine, 10 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide or 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc). The enzyme was stable in the borate buffer for 24 h at 0 or 24 °C.

After affinity chromatography only about 45–50 % of the *N*-acetyl-β-D-hexoaminidase A activity was recovered. However, the activity could be stimulated 60–80 % by recombining the enzyme with the non-enzymatic protein portion (dotted line on Fig. 1) suggesting that much of the activity loss may have been due to removal of a non-enzymatic component of the urinary proteins which exerted a stimulatory effect. When assayed in the presence of 0.1 % bovine serum albumin the enzyme was also stimulated 45–55 %.

Polyacrylamide gel electrophoresis of the A isozyme fraction after affinity chromatography showed it still to be heterogeneous with between three and six minor protein bands. However, at least six other bands which were present in the starting material had been removed. The banding pattern of the starting material and that of the unadsorbed non-enzymatic proteins was identical, suggesting that the contamin-

ating proteins in the enzyme fraction were either residual amounts of some of the unadsorbed non-enzymatic proteins or were additional minor components originally masked by the larger amounts of those proteins.

When the *N*-acetyl-β-D-hexosaminidase B fraction was applied to the affinity column under conditions identical with those described above, the enzyme was not tightly bound, but rather only slightly retarded (Fig. 2). The smaller second peak of

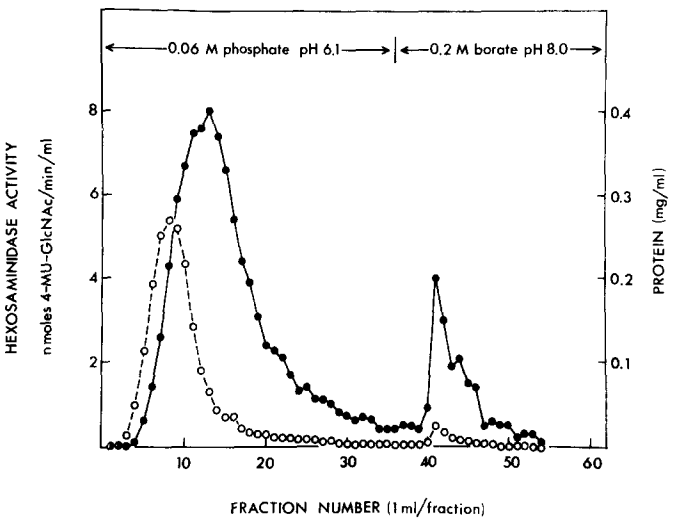

Fig. 2. Affinity chromatography of partially purified *N*-acetyl-β-D-hexosaminidase B. 4.8 ml of a partially purified *N*-acetyl-β-D-hexosaminidase B preparation containing approx. 1.8 mg protein was applied to a small affinity column. Experimental conditions were as described in Fig. 1. Solid line represents *N*-acetyl-β-D-hexosaminidase activity, dotted line represents protein.

enzyme activity which eluted with borate may have been some contaminating A form but has not been identified. The difference in affinity for the A and B forms was unexpected, considering the similar activity of the two isozymes towards the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, which the ligand resembles in structure.

An attempt was made to isolate the A isozyme from crude urinary proteins without prior purification. Fresh urine was filtered through a glass fiber filter and protein was precipitated with 100% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 0.05 M phosphate buffer, pH 6.1, and dialyzed overnight against the same buffer. Chromatography of the retentate on the affinity adsorbent removed *N*-acetyl-β-D-hexosaminidase B and about 90% of all contaminating protein. *N*-Acetyl-β-D-hexosaminidase A activity was recovered in 50% yield, with a 4-fold purification.

Experience with both crude and partially purified enzyme shows that the adsorbent can be used for a significant purification of *N*-acetyl-β-D-hexosaminidase A, but that it must be combined with other procedures if a homogeneous product is desired. While this work was in progress, an affinity chromatography system for *N*-acetyl-β-D-hexosaminidase A, using a naturally-occurring glycopeptide as a ligand, has been developed by Dawson et al. [14]. Combined use of both systems may prove especially useful for extensive purification of the enzyme on a microscale, as may be required when only fibroblasts or biopsy specimens are available for investigation.

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