Journal of Chromatography, 136 (1977) 141–146

CHROM. 9875

PURIFICATION OF β-N-ACETYL-D-GLUCOSAMINIDASE OF THE HORSESHOE CRAB BY AFFINITY CHROMATOGRAPHY

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(Received December 13th, 1976)

SUMMARY

An affinity column has been synthesized consisting of *p*-aminophenyl 1-thio-β-L-fucopyranoside residues attached to Sepharose 4B through succinylated diaminodipropylamine bridges. Surprisingly, it has been found to bind β-N-acetylglucosaminidase in the serum of *Limulus polyphemus* (horseshoe crab). The enzyme is eluted with N-acetyl-D-glucosamine at a concentration of 2 mg/ml and with other sugars at higher concentrations. A highly purified enzyme free from other glycosidases is obtained. The enzyme is not eluted by solutions of salt.

INTRODUCTION

In an attempt to see whether β-L-fucosidase exists in nature, an affinity column was designed that might bind the enzyme so that it could be purified and concentrated before being assayed. The column was made by attaching residues of *p*-aminophenyl 1-thio-β-L-fucopyranoside to Sepharose 4B through succinylated diaminodipropylamine bridges. The assay was based upon the splitting of *p*-nitrophenyl β-L-fucopyranoside. This enzyme, if it exists, could be exploited to see whether L-fucopyranoside is bound in β-linkage, especially in situations where bound L-fucose is found to be resistant to α-L-fucosidase from various biological sources. We could find no β-L-fucosidase in a wide variety of marine invertebrates and plants. However, we found that the column material bearing the β-thiofucopyranoside residues bound β-N-acetyl-D-glucosaminidase (E.C. 3.2.1.30) rather specifically and could be used effectively for its purification. This enzyme has been previously purified on an affinity column bearing residues of *p*-aminophenyl-N-acetyl-1-thio-β-D-glucosaminide[1,2].

EXPERIMENTAL

All materials used were reagent-grade commercial samples, unless otherwise

Synthesis of p-aminophenyl 1-thio-β-L-fucopyranoside

The procedure of Janaki *et al.*[3] was used for the synthesis of the β-anomer of *p*-aminophenyl 1-thio-L-fucopyranoside. Briefly, for the β-anomer the acetylated α-L-fucopyranosyl bromide was condensed with *p*-nitrothiophenol in the presence of potassium hydroxide. The resulting *p*-nitrophenyl 1-thio-β-L-fucoside was reduced with hydrogen over platinum oxide (Adam's catalyst) to obtain the desired 1,2-*trans*-(β)-*p*-aminophenyl 1-thio-L-fucopyranoside.

p-Nitrophenyl 1-thio-β-L-fucopyranoside

A solution of 10.2 g (0.029 mole) of the syrupy bromide prepared from 1,2,3,4-tetra-O-acetyl-L-fucopyranose[1] in dry (calcium chloride) chloroform (50 ml) was added dropwise to a stirred solution of 4.65 g (0.03 mole) of *p*-nitrothiophenol in methanolic potassium hydroxide (100 ml, 0.5 *M*) kept at 0° in a dry nitrogen atmosphere. After 6 h at room temperature under nitrogen, the reaction mixture was left in the refrigerator overnight. Ice–water was added to dissolve the precipitated potassium bromide. On adding a little more water, a light yellow solid was crystallized out. The solid was filtered, washed with diethyl ether to give 2.1 g (24.1%) of the desired product. M.p. 177–178°, lit.[4] 177–178°; $[\alpha]_D^{24}$ + 122.85° (C = 1, ethanol), lit.[4], + 116.0 (C = 1, methanol). Recrystallization from ethyl acetate did not improve the melting point. The compound was spectrally pure, by IR[4]; NMR δ 8.12 (d, 2, J = 8 Hz, $H_{o\text{-nitro}}$); 7.58 (d, 2, J = 8 Hz, $H_{m\text{-nitro}}$); 3.05–4.48 (complex multiplet, 10) and 1.22 ppm (d, 3, J = 6 Hz). Analytical calculations for $C_{12}H_{15}NO_6S$: C, 47.84; H, 5.02; N, 4.65%. Found: C, 47.41; H, 4.59; N, 4.57%. The chloroform layer, on further work-up, gave 1.75 g (20%) of the impure product; m.p. 148–165°.

p-Aminophenyl 1-thio-β-L-fucopyranoside (Fig. 1)

The β-L-thiofucopyranoside (1 g) was hydrogenated for 2 h with platinum oxide (Adam's catalyst) (0.1 g) in freshly distilled ethanol (100 ml) at an initial pressure of 40 lb/in.^{2}. The catalyst was filtered off, the solvent was removed on a rotary evaporator and the residue was washed several times with diethyl ether and light petroleum to obtain a colorless crystalline solid. Yield, 0.75 g (83%); m.p. near 70°, softens before melting (lit.[4], 65–78°); $[\alpha]_D$ +79.15° (C = 1, ethanol), lit.[4], $[\alpha]_D$ +66.0; R_F 0.8 (ethyl acetate–acetic acid–water, 3:1:1; silica gel plate). The compound was spectrally pure; IR and NMR showed it to be identical with the α-L-anomer[4]. The resin was washed with 100 ml of methanol. Removal of the solvent on a rotary evaporator yielded 0.5 g (87%) of the desired product. The product was triturated with diethyl ether according to Chawla and Bahl[4] and filtered, yielding 0.45 g of slightly yellow crystals; m.p. 218–226° (lit.[4] 224–226°); $[\alpha]_D$ −343° (methanol), lit.[4], −382°; R_F 0.85 (lit.[4], R_F 0.88).

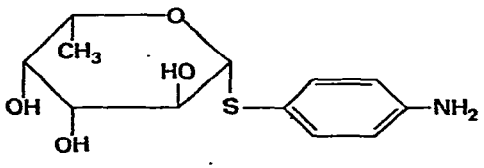

Fig. 1. Structure of *p*-aminophenyl 1-thio-β-L-fucopyranoside.

Preparation of affinity columns

Sepharose 4B was activated with cyanogen bromide, coupled with 3,3'-diaminodipropylamine according to the method of Cuatrecasas[5]. Succinylation of the 3,3'-diaminodipropylamine Sepharose 4B was also effected by the method of Cuatrecasas[5]. To couple *p*-aminophenyl 1-thio-β-L-fucopyranoside to the succinyl derivative, 0.068 g of the fucoside was dissolved in 1 ml of dimethylformamide and added to 10 ml of the Sepharose derivative suspended in water. The pH was adjusted to 4.7 then 0.5 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 1 ml of water was added dropwise. The pH was maintained at 4.7 for 1 h. The suspension was stirred at room temperature overnight and was then washed with 4 l of 0.1 *N* sodium chloride solution in a Büchner funnel (coarse disc) without using suction. Columns were stored in 0.1 *N* sodium chloride solution containing 0.1% sodium azide at 4°.

Glycosidases were assayed essentially by the method described by Bosmann[6]. A typical incubation mixture contained 0.3 ml of 0.05 *M* sodium citrate (pH 4.5), 0.3 μmole in 0.3 ml water of *p*-nitrophenylglycoside substrate, and 0.05 ml of a solution of enzyme. When α-mannosidase was assayed, 1.2 μmole of the *p*-nitrophenyl α-mannoside substrate was used. After 20 min of incubation at 37°, the vessels were chilled in ice and 0.6 ml of 0.2 *M* potassium borate buffer (pH 9.8) was added and the absorbance was determined at 400 nm. A unit of enzyme can cleave 1 μmole of substrate in 1 h under the conditions of the assay. Specific activities are expressed as units per milligram of protein.

For routine purifications, an 11 × 0.8 cm column was used. The column material was equilibrated with 0.2 *M* sodium citrate buffer (pH 6.0) containing 0.02% sodium azide. In most runs, 0.5 ml of *Limulus* serum was added to the column and buffer was added until the absorbance at 280 nm approached zero (20 ml). The enzyme was then eluted with 5 ml of buffer containing 10 mg of N-acetyl-D-glucosamine or other sugars, depending upon the experiment. The flow-rate was approximately 0.3 ml/min. The buffer containing the enzyme and sugar was dialyzed overnight against 0.02 *M* sodium citrate buffer (pH 6.0). As the same results (yields, purification) were obtained on running the column at 4° or at room temperature, experiments were carried out at the latter temperature. However, the purified enzyme was stored at 4°, at which temperature it was completely stable for several months.

RESULTS

The relative activities of various glycosidases of *Limulus* serum* are given in Table I. β-N-Acetyl-D-glucosaminidase activity is present in a considerably greater amount than that of other glycosidases. α-L-Fucosidase, usually present in natural materials in small amounts, is surprisingly active and has been purified by affinity chromatography[7].

When serum of *Limulus* is placed on a column bearing ligands of *p*-aminophenol 1-thio-β-L-fucopyranoside, the bulk of the protein and glycosidases pass through, including α-L-fucosidase. After extensive washing of the column, a solution

* The serum was obtained at the Marine Biological Laboratory, Woods Hole, Mass., U.S.A., and some of the work using serum of *Limulus* was also carried out there.

TABLE I

ACTIVITY OF VARIOUS GLYCOSIDASES IN SERUM OF *LIMULUS POLYPHEMUS*

Glycosidase	*Activity (U/ml)*	*Specific activity (μmole/mg·h)*
α-L-Fucosidase	2.66	0.0242
β-L-Fucosidase	>0.01	>0.0001
β-D-Fucosidase	0.07	0.0006
α-D-Galactosidase	0.14	0.0013
β-D-Galactosidase	0.12	0.0011
β-N-Acetyl-D-galactosaminidase	0.69	0.0063
β-D-Glucosidase	0.11	0.0010
β-N-Acetyl-D-glucosaminidase	6.10	0.0550
α-D-Mannosidase	2.33	0.0210

of L-fucose in buffer displaces a small amount of protein, as well as a relatively large amount of β-N-acetyl-D-glucosaminidase activity.

N-Acetyl-D-glucosamine is used routinely for elution of enzyme from the column. It can be seen in Fig. 2 that at 2 mg/ml of buffer, maximum elution of enzyme takes place. At 40 mg/ml, various sugars, including N-acetyl-D-glucosaminitol, D-mannose, D-glactose, L-fucose, D-glucose and sorbitol, are as effective as N-acetyl-D-glucosamine in displacing β-N-acetyl-D-glucosaminidase, while at the lower concentration of 2 mg/ml they elute only trace amounts of this enzyme activity. The enzyme is not eluted by 1 *M* potassium chloride solution or by shifts of pH down to 3.5. The enzyme is not retained by Sepharose 4B bearing succinylated 3,3'-diaminodipropylamine spacers.

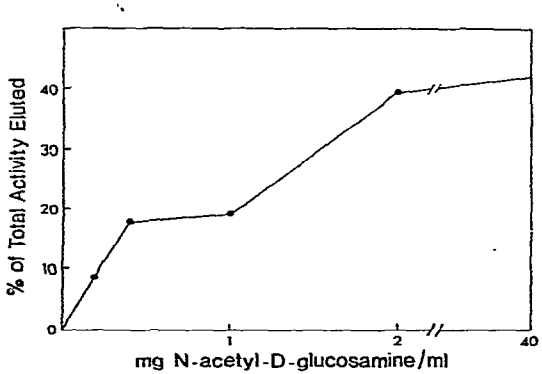

Fig. 2. Assay for β-N-acetyl-D-glucosaminidase as described in the text.

Not all of the β-N-acetyl-D-glucosaminidase applied binds to the column. Approximately 15% of the enzyme activity applied passes through, while 40% of that applied can be eluted with L-fucose in purified form (130-fold purification). Almost half of the original activity is unaccountably lost; it cannot be removed by high concentrations of L-fucose or of sodium chloride. The fraction of activity not binding to the column is the same regardless of how rapidly serum is passed through, and the percentage that binds does not vary when the enzyme is applied over a 10-fold

range of concentration (0.1–1.0 ml of serum per 4 ml of column material). Surprisingly, if the material that has passed through the column is recycled on the column, 30% of the N-acetyl-D-glucosaminidase activity is bound and approximately half of the enzyme can be eluted by N-acetyl-D-glucosamine. When the enzyme that was originally bound and eluted by N-acetyl-D-glucosamine is passed over the column a second time, 99% is bound and 85% of the bound activity can be recovered. It may be that the enzyme exists in two forms, a non-binding type that can be converted into a binding form; the reverse transition may occur but slowly or not to a great extent. The β-N-acetyl-D-glucosaminidase of rat epididymus, *Aspergillus* and jack bean does not adhere to the column under any circumstances.

The purified enzyme, over 100-fold purified with a specific activity of 4–7, is essentially free from proteinase (Azocoll assay), α- and β-D-galactosidase, α-D-mannosidase, α-L-fucosidase, α-D-glucosidase and β-D-glucosidase. Only one band is seen when the enzyme is subjected to electrophoresis in a sodium dodecyl sulfate–polyacrylamide system and stained with Coomassie blue.

β-N-Acetyl-D-galactosaminidase is found in *Limulus* serum in about 10% of the concentration of β-N-acetyl-D-glucosaminidase. Its behavior on the column is erratic, sometimes being eluted by various sugars (40 mg/ml) and at other times not. N-Acetyl-D-galactosamine was no more effective or predictable in eluting the enzyme from the column than was N-acetyl-D-glucosamine. Most of the activity could be eliminated from serum by preliminary precipitation with solid ammonium sulfate; 81% of the enzyme could be precipitated between 0 and 45% saturation (4°, 4 h stirring). Over 60% of the β-N-acetyl-D-glucosaminidase remains in the supernatant solution and can be precipitated by raising the concentration of ammonium sulfate to 60% saturation. In two experiments it was found that D-mannose (40 mg/ml) eluted β-N-acetyl-D-glucosaminidase but not β-N-acetyl-D-galactosaminidase.

Characteristics of the enzyme

The pH optimum of the enzyme is 4.8. While activity is reduced by only 23% at pH 6–7, there is a precipitous drop at pH values lower than 4.5.

The K_m values of the assay substrate, *p*-nitrophenyl-β-N-acetyl-D-glucosamine for enzyme that adhered to the column and that which passed through, were found to be $4.5 \cdot 10^{-4}$ and $3.1 \cdot 10^{-4}$ *M*, respectively. The K_i values of *p*-aminophenyl 1-thio-β-L-fucopyranoside for the two forms of the enzyme were $1.8 \cdot 10^{-3}$ *M* for the enzyme that did not adhere and $5.5 \cdot 10^{-4}$ *M* for the enzyme that adhered. The thio derivative is a very weak competitive inhibitor which has a somewhat higher affinity for the enzyme that binds to the column.

DISCUSSION

There appears to be no obvious explanation why β-N-acetyl-D-glucosaminidase should adhere in a rather specific manner to a column bearing β-L-thiofucopyranoside residues. Although salt solutions and variations of pH did not alter the binding, several polyhydroxylated compounds were capable of eluting the enzyme at a concentration of 40 mg/ml. However, N-acetyl-D-glucosamine was the only sugar capable of effectively eluting the enzyme at relatively low concentration (2 mg/ml). Ring structure, a 6-methyl or precise configuration of the hydroxyl groups were not

important factors at high sugar concentrations. Perhaps β-L-fucosides do not, in fact, exist in nature because they would be confused with β-N-acetylglucosaminides. The basis for this possible cross-specificity is unknown.

The column has been found useful for the purification of β-N-acetyl-D-glucosaminidase from the serum of the horseshoe crab. In addition, it appears to distinguish between at least two forms of the enzyme, one that adheres and a second that passes through the column.

ACKNOWLEDGEMENTS

This work was supported by USPHS research grants CA 13992 and CA 14985 from the National Cancer Institute, PRP-28 from the American Cancer Society and a grant from the National Science Foundation. The expert assistance of Ms. K. Malmstrom is gratefully acknowledged.

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