PURIFICATION OF *N*-ACETYL D-GLUCOSAMINE-BINDING PROTEINS BY AFFINITY CHROMATOGRAPHY

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1. Introduction

Highly purified osidases are required for structural studies of oligosaccharides. Affinity chromatography [1] is one of the best techniques which can be used to reach this purpose [2–5]. Affinity chromatography utilizes the highly specific and strong interactions which take place between many pairs of complementary molecules. Specific matrices are prepared by attaching ligands such as competitive inhibitors, natural enzyme inhibitors, cofactors or substrates. This paper describes the preparation of a hexosaminidase inhibitor and its use for the purification of this osidase by affinity chromatography. The same matrix can be used for the isolation of the wheat germ *N*-acetyl glucosamine-binding protein.

2. Materials and methods

2.1. *Synthesis of p-aminobenzyl 1-thio 2-acetamido 2-deoxy β-D-glucopyranoside (V)*

This compound was prepared by a 4-step synthesis (fig. 1). Step 1: reaction of thiourea with 2-acetamido 2-deoxy 3,4,6-tri-*O*-acetyl α-D-glucopyranosyl chloride (I) [6] led to 2-(2-acetamido 2-deoxy 3,4,6-tri-*O*-acetyl β-D-glucopyranosyl)-2-thiopseudourea hydrochloride (II); step 2: reaction of *p*-nitrobenzylbromide with II led to *p*-butrobenzyl 1-thio 2-acetamido 2-deoxy 3,4,6-tri-*O*-acetyl β-D-glucopyranoside (III) [7]; step 3: catalytic desacetylation of (III) led to *p*-nitrobenzyl 1-thio 2-acetamido 2-deoxy β-D-glucopyranoside (IV); step 4: catalytic reduction of (IV) led to (V).

Compound II

I (3.65 g: 10 mM; Koch Light) and thiourea (0.76 g: 10 mM; Merck) were refluxed for 30 mn with 10 ml of dry acetone. After cooling to 4°C. the insoluble product (II) was filtered and washed with cold acetone (yield: 3.55 g; 80%). Recrystallization from ethanol gave white needles (yield: 3.1 g; 70%).

Compound III

A solution of potassium carbonate (0.8 g: 5.7 mM) and sodium dithionite (1 g: 5.7 mM) in water (10 ml) was dropped into a solution of compound (II) (2.21 g: 5 mM) and *p*-nitrobenzyl bromide (2.16 g: 10 mM) in acetone (10 ml). This solution was stirred continuously for 30 min. An equal volume of water was added and the compound extracted with chloroform. The organic layer was washed successively with diluted sulfuric acid (5%), with saturated bicarbonate aqueous solution and with water. The final chloroform solution was dried over anhydrous sodium sulfate, filtered and concentrated. Compound III was isolated by silica gel (70-325 mesh, Merck) column chromatography using the solvent system chloroform/ethanol (9:1; v/v) and then crystallized from ethyl acetate (yield 1.27 g: 52%).

Compound IV

To a solution of compound III (500 mg: 1 mM) in anhydrous methanol (10 ml) was added a solution of sodium methoxide 0.4 N in methanol (0.5 ml). The mixture was kept overnight at 4°C. Compound IV was obtained after silica gel column chromatography with the solvent system ethyl acetate/methanol (3:1; v/v) (yield 370 mg, 100%).

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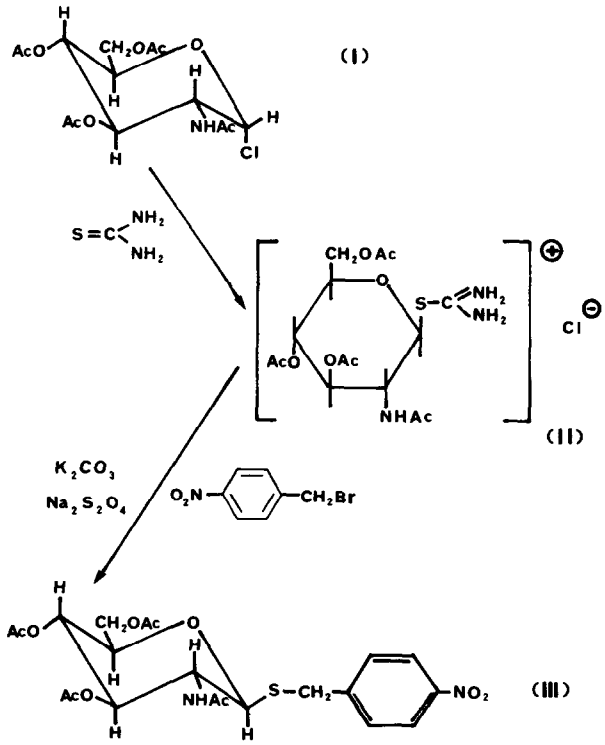

Fig. 1. Synthesis of *p*-nitrobenzyl 1-thio 2-acetamido 2-deoxy 3,4,6-tri-O-acetyl β-D-glucopyranoside (III).

Table 1
Optical rotation; melting points; infrared and nuclear magnetique resonance characteristics for compounds II, III and IV

	m.p. $[\alpha]_{589}^{25°}$	ν_{max}^{KBr} cm^{-1}	NMR
II*	186°–188°C −27.7 (C 1, methanol)	3200 (NH); 3030 (C=NH_2^+); 1750 (C=O); 1650 (amide I); 1550 (amide II); 1240 (C–O); 700 (C–S).	D_2O; τ 3.22 (ld, J: 4 Hz, H–1); 7.18 (ls, acetamido); 7.30, 7.32, 7.33 (3s, 3 acetyl).
III	218°–219°C −122.4 (C l, chloroform)	3300 (NH); 3080, 2940, 2870 (CH_2); 1750 (C=O); 1665 (amide I); 1560 (amide II); 1530 (NO_2); 1355 (NO_2); 1230 (C–O); 860 (NO_2); 830 (C–H ar); 720 (C–S).	$CDCl_3$; τ 1.92 – 2.46 (2d, J: 9 Hz, benzyl); 4.07 (ld, J: 8 Hz, H-1); 6.29 (1 *m*, H-5); 7.45 – 7.96 (4s, 1 acetamido, 3 acetyl).
IV	234°–236°C −132.4 (C l, dimethylformamide)	3300 (OH,NH); 2950, 2870 (CH_2); 1660 (amide I); 1560 (amide II); 1530, 1355, 865 (NO_2); 830 (CH ar); 715 (C–S).	Dimethylsulfoxide d_6; τ 1.77 – 2.32 (2d, J: 9 Hz, benzyl); 7.94 (ls, acetamido).

Melting points were taken between glass slides on a Leitz apparatus. Rotations were determined with a Perkin–Elmer Model 141 polarimeter. Infrared spectra were determined on KBr pellets with a Perkin–Elmer Model 257 spectrometer. NMR spectra were recorded with a 90 MHz Bruker spectrometer using chloroform-d_6, dimethylsulfoxide-d_6 and deuterium oxide as solvents, with tetramethylsilane as internal standard for III and IV and external standard for II.

* Compound II was first synthetized by Horton and Wolfrom: m.p.: 179°–181°C; $[\alpha]_{589}^{22}$ – 29.2 (C l, methanol) [8].

Compound V

The nitroderivative IV (446.4 mg: 1.2 mM) dissolved in methanol was reduced by action of hydrogen (0.1 Atm, 7 hr) with 0.5 g of palladium charcoal as catalyser. The formation of V was monitored by silica gel G (Merck) thin layer chromatography with the solvent system chloroform/methanol (2:1; v/v).

The overall yield of derivative IV from I was about 40%. The analytical data for compounds II, III, and IV are summarized in table 1. The NMR characteristics indicated that compounds II, III and IV are the β anomers.

2.2. *Protein extraction and enzyme activity*

The osidases: β-*N*-acetylglucosaminidase (β-2-acetylamino-2-deoxy-D-glucoside acetylaminodeoxyglucohydrolase, EC 3.2.1.30); β-galactosidase (β-D-glactoside galactohydrolase, EC 3.2.1.23) and α-mannosidase (α-D-mannoside mannohydrolase, EC 3.21.24) were extracted from Jack bean meal (Sigma: J 0 125) according to Li and Li [9] and precipitated with ammonium sulfate [10]. The precipitate was solubilized in sodium phosphate buffer (0.05 M pH 7.0). This solution could be kept several weeks at 4°C.

The glycosylhydrolase activities were monitored by using *p*-nitrophenyl glycosides according to Conchie [11]. No β-thioglucosamidyl hydrolase activity was found using compound IV as a substrate according to the method described by Reissig et al. [12] and modified by Levy and McAllan [13] to detect free *N*-acetylglucosamine.

The wheat germ agglutinin (WGA) was extracted according to the method of Levine et al. [14] modified by Mialonier [15] in our laboratort. After ammonium sulfate fractionation, the solution was chromatographed on Sephadex G-50 and then on carboxymethyl cellulose columns. The fraction containing WGA and *N*-acetylglycosaminidase were pooled and chromatographed on the affinity column.

2.3. *Affinity chromatography*

Sepharose 4B (Pharmacia, Sweden) (25 ml) activated with cyanogen bromide (Porath et al. [16]) was coupled with hexamethylenediamine, treated with succinic anhydride and then activated with CMCI [*N*-cyclohexyl-*N'*-(2-(4-β-morpholinyl)-ethyl) carbodiimide-methyl-*p*-toluene sulfonate] [17]. Compound V (171 mg: 0.5 mM) was linked immediately after the

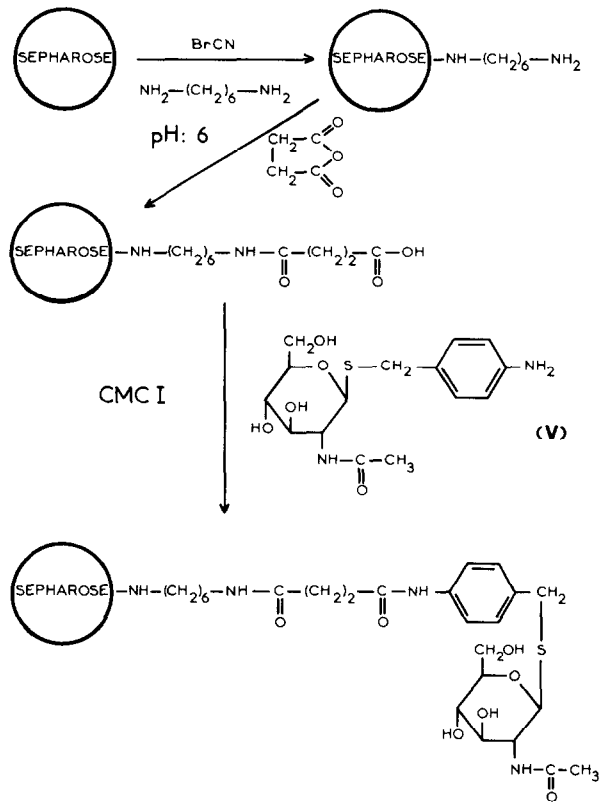

Fig. 2. Preparation of the affinity chromatography adsorbent.

reducing step to this activated Sepharose 4B (fig. 2). In order to avoid any ion exchange effects, unreacted carboxyl groups in the extension arm were blocked with 0.5 mM of Tris [2-amino 2 hydroxymethyl propane 1,3-diol] and with 1.6 mM of CMCI. The substituted sepharose was washed with a 0.05 M phosphate–citrate buffer (pH 5.8) and poured into a column (i.d. 1.5 cm).

3. Results and disccussion

The hexosaminidase activity showed a maximum between pH 4.8 and 5.8, but was very low at pH 7.0; dialysis overnight at pH 4.8 resulted in 50% loss of activity, but no loss activity was found by dialysis at pH 5.8. The affinity chromatography was carried out at pH 5.8.

The K_M value (Michaelis constant) for the reaction

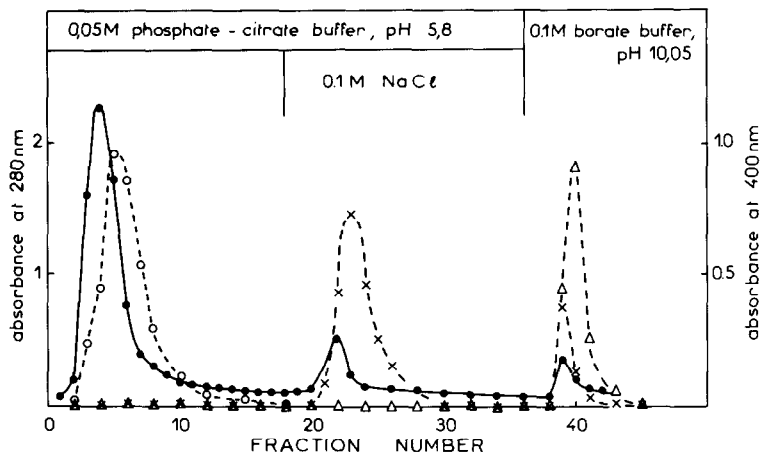

Fig. 3. Separation of β-galactosidase, β-*N*-acetylglucosaminidase and α-mannosidase on affinity chromatography column (1.5 × 15 cm; flow rate: 10 ml/hr). The enzymatic solution was dialysed overnight against a 0.05 M phosphate–citrate buffer (pH 5.8; 0.1 M; citric acid 19.7 ml; 0.2 M; disodium phosphate 30.3 ml; 50 ml distillated water). This enzymatic solution, containing the β-hexosaminidase (20–100 e.u.) was applied at the top of the column. After extensive washing with pH 5.8 phosphate–citrate buffer, elution was started with a same buffer containing 0.1 M NaCl (200 ml), then, with 0.1 M sodium borate buffer pH 10.05 (100 ml). Nine milliliters fractions were collected. The proteins were monitored by 280 nm absorbance determination (●–●–●) and the osidase activities by 400 nm absorbance determination with 50 μl fractions for β-galactosidase (○–○–○); with 10 μl fractions for β-*N*-acetylglucosaminidase (x–x–x) and α-mannosidase (Δ–Δ–Δ).

of J.B.M. hexosaminidase on *p*-nitrophenyl 2-acetamido 2-deoxy- β-D-glucopyranoside was 2.5×10^{-4} M while the K_i value (inhibition constant) for reaction on compound IV was 10^{-3} M. Although this constitued a low affinity system, the sepharose ligand was very efficient for the purification procedure. Using *p*-aminobenzyl l-thio β-D-galactopyranoside, Steers et al. [3] were successful in isolating β-D-galactosidase although the K_i value was still lower.

In control experiments, a protein sample of Jack bean meal was chromatographed on unsubstituted Sepharose, on aminohexylaminylsepharose and on succinyl-aminohexylaminylsepharose. The glycosylhydrolases were eluted with the frontal protein peak. No proteins were eluted when borate solution was applied. On the contrary, when the inhibitor substituted sepharose was used, α-mannosidase and hexosaminidase activities were retained whereas β-D-galactosidase was eluted with the other inactive proteins. The two glucosylhydrolases retained could not be desorbed by increasing the pH until 9.5 but were eluted together by using 0.1 M sodium borate buffer pH: 10.05. The hexosaminidase was not desorbed by *N*-acetylglucosamino solution up to 0.2 M, but was easily eluted by increasing the ionic strength of the buffer (fig. 3). About 65% of the activity was recovered (hexosaminidase I). A further elution with 0.1 M sodium borate buffer pH 10.05 led to the elution of a mixture of hexosaminidase (II) (about 10%) and of all the α-mannosidase.

The fractions corresponding to hexosaminidase I and II showed the same activities with respect to the hydrolysis of *N*-acetylglucosaminidine and *N*-acetylgalactosaminidase (activity ratio: 1.50).

The abnormal behavior of α-mannosidase and the strong adsorption of a small amount of hexosaminidase II were not understood.

The purification factor for the hexosaminidase was about 150 with a recovery of 60–70%.

The hexosaminidase from wheat germ behaved similarly to that of J.B.M. on this affinity column. Moreover the *N*-acetylglucosamine-binding agglutinin (WGA) was quantitatively retained and could be selectively eluded by 0.1 M acetic acid. Detailed studies of the purification of WGA will be described elsewhere.

Acknowledgements

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