PURIFICATION OF *N*-ACETYL β-D-HEXOSAMINIDASE FROM BULL EPIDIDYMIS BY AFFINITY CHROMATOGRAPHY

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1. Introduction

N-Acetyl β-D-hexosaminidase (β-2-acetamido-2-deoxy-D-glucopyranoside acetylaminodeoxyglucohydrolase, E.C. 3.2.1.30, sometimes called β-acetylglucosaminidase) is widely distributed among living organisms [1]. It is an *exo*-enzyme and catalyses the hydrolysis of *N*-acetyl β-D-glucosyl- and *N*-acetyl β-D-galactosylamine and adjacent sugars or aglycones [2]. It plays an important role in the metabolism of glycosphingolipids [3]. Our laboratory is interested in this enzyme and has shown that synthetic 2-acetamido-2-deoxy-D-glucono-1,4-lactone (1) and the corresponding 2-acetamido-2-deoxy-D-galactono- (2), and 2-acetamido-2deoxy-D-mannono-1,4-lactones (3) exhibit inhibitory activity toward the *N*-acetyl-β-D-hexosaminidase from Bull epididymis [4,5,6]. In order to obtain the pure enzyme so that studies can be undertaken on its binding behavior with various ligands by quantitative methods [7], affinity chromatography using either of the three inhibitors mentioned above, coupled to cyanogen bromide-activated Sepharose, was attempted.

Several other workers have recently used affinity chromatography to purify *N*-acetyl hexosaminidase from human skin fibroblasts [8], urine [9], rat liver [10] and Jack Bean meal [11]. The affinity ligands used were a glycopeptide, *p*-aminophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, *p*-aminophenyl-1-thio-2-acetamido-2deoxy-β-D-glucophranoside, respectively. In all these cases an approximately 150-fold purification was achieved in one step. Isolation of *N*-acetyl hexosaminidase from human placenta [12] was achieved in a higher yield by using 2-acetamido-*N*-(ε-aminocaproyl-2-deoxy-D-glucopyranosylamine).

We wish to report the purification of this enzyme from Bull epididymis by use of the specific hexosaminidase inhibitors, 2-acetamido-2-deoxy-D-glucono-1,4-lactone (1) and the corresponding D-galactono- (2) and D-mannono (3) lactones, coupled to cyanogen bromide-activated Sepharose by a benzidine bridge [13].

2. Materials and methods

Bull epididymises were obtained from Pel-Freez Biologicals Inc. (Arkansas, Ark. 72756 U.S.A.) and stored at −15°C until required. p-Nitrophenylglycosides, and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride were obtained from Sigma Chemical Company (St. Louis, Mo. USA). Crystalline 2-acetamido-2-deoxy-D-glucono-1,4-lactone, 2-acetamido-2-deoxy-D-galactono-1,4-lactone as well as 2-acetamido-2-deoxy-D-mannono-1,4-lactono were prepared as already described [4,14]. Sepharose 4B was supplied by Pharmacia Fine Chemicals, Sweden. A Beckman Acta V spectrophotometer was used for the u.v. measurements.

2.1. *Enzyme extraction and essay*

N-Acetyl β-D-hexosaminidase was extracted from Bull epididymis according to the method of Levvy and Conchie [15]. The fraction which precipitated between 20–70% saturation with ammonium sulfate was retained, and this was dissolved in a small volume of 50 mM sodium phosphate–citrate buffer (pH 5.5). Dialysis against the same buffer gave a stock solution containing 2 Eu per mg of protein, when tested with *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyrano-

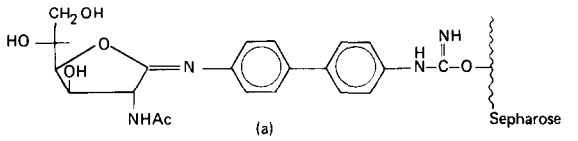
side as follows: the incubation mixture (2.0 ml) containing the above glycoside (2.5 mM) and a suitable amount of enzyme in sodium phosphate–citrate buffer (pH 4.8) were incubated at 38°C for 15–20 min. (In the case of the *N*-acetyl-β-galactosaminidase assay, 0.5 mM *p*-nitrophenyl-2-acetamido-2-deoxy-D-galactopyranoside in the same buffer at pH 4.4 was used). Glycine–sodium hydroxide buffer, pH 10.5, (0.4 M, 5 ml) was added and the liberated *p*-nitrophenol was determined spectrophotometrically at 430 nm. Total protein concentration was monitored during purification procedures either by measuring the absorbance at 280 nm, or colorimetrically, by Lowry's method [16]. Crystalline BSA (Sigma) was used as a standard.

2.2. *Affinity chromatography*

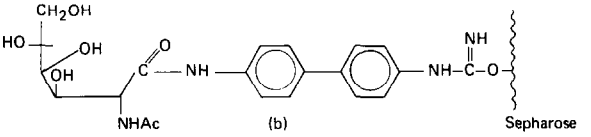
Sepharose 4B was converted into the active imidocarbonate derivative with cyanogen bromide [17], and benzidine was coupled to this reactive matrix as described by Kanfer et al. [13]. Settled derivated gel (100 ml) was suspended in water (100 ml) and the required lactone (2.0 g) was added with stirring. To this mixture was added 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (500 mg) and the suspension was stirred for 3 days. The pH was adjusted to 4.0 every 12 hr. The resulting ligand containing gel was washed on the filter with water (1 litre) and treated with 100 ml water containing acetic anhydride (2 ml) while being sonicated 5 times for 1 min. It was then washed on a filter with 50 mM phosphate citrate buffer, pH 5.5, and kept cold until required. So prepared, it contained 2.7–2.9% N. A 2 × 13 cm column could be loaded with 50 mg of protein without saturation. After high pH elution of the enzyme (see below) the column could be recycled by washing with 50 mM phosphate citrate buffer, pH 5.5.

3. Results and discussion

Compounds (1), (2) and (3) recently isolated in crystalline form [4,14] were each coupled to Sepharose 4B bearing a benzidine side arm [13] under identical conditions. Whether the linkage formed between the lactone and the benzidine-derivatized Sepharose is an imino ester (a) or an amide (b) is not known to us at this time.



(a)



(b)

Imino esters are stable under aqueous conditions, form hydrochlorides and can be regenerated by alkali. It is therefore, likely that they would be stable under our conditions, once formed. The ring form is a prerequisite for inhibitory activity, and the affinity ligands do cause binding of the enzyme to the matrix. It may therefore be possible that structure (a) is the one responsible for the binding. In any case, when an enzyme extract from Bull epididymis (partially purified by ammonium sulfate precipitation) was passed through any of the three affinity columns, *N*-acetyl β-D-hexosaminidase was selectively retarded on the column irrespective of which of the three lactones, (1), (2) or (3), had been coupled to benzidine–Sepharose 4B. The enzyme could be eluted from the column by 0.2 M borate buffer, pH 8.0; 0.08 M McIlvaine buffer, pH 8.0; or 1% Triton X-100 in McIlvaine buffer, pH 5.5. Best results were obtained with the borate buffer, but as 0.2 M NaCl in 50 mM McIlvaine buffer, pH 5.5., eluted some proteins not showing enzyme activity from the column, we found it useful to wash the column with this eluant prior to elution with borate buffer.

Four other glycosidases initially present in the partially purified epididymis extract (β-galactosidase, α-mannosidase, β-glucuronidase and α-galactosidase) were not bound by the column when 0.05 M phosphate–citrate buffer was used, and were eluted in the first peak (Fig.1). *N*-Acetyl β-D-hexosaminidases are usually resolved with difficulty from other glycosidases by conventional purification procedures. This method of separation therefore offers great advantages.

The specific purification, yields, as well as ratios between *N*-acetyl β-D-glucosaminidase and *N*-acetyl

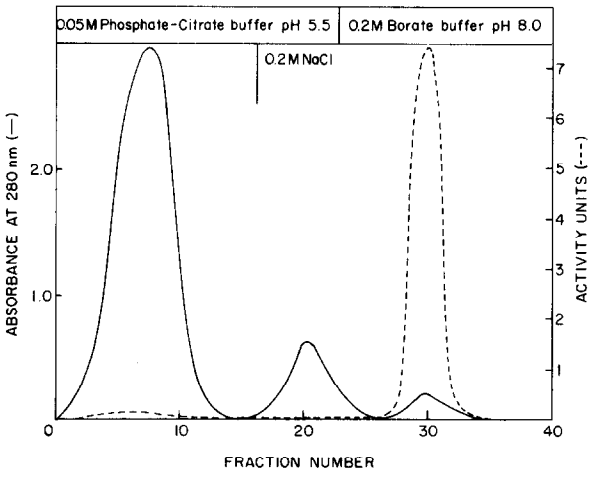


Fig.1. Elution pattern of 2-acetamido-2deoxy-β-D-hexosaminidase from affinity column (2 × 13 cm); flow rate 12 ml/hr, 10 ml fractions were collected. Enzymatic solution (50 ml containing 20 enzyme units, or 10 mg of proteins) previously dialyzed against 0.05 M sodium phosphate-citrate buffer pH 5.5 were applied to the column. The column was washed first with the same buffer (150 ml), then with the buffer containing 0.2 NaCl (100 ml) and lastly with 0.2 M boric acid–borax buffer pH 8.0.

β-D-galactosaminidase activity are given in table 1. A number of observations can be made. The use of 2-acetamido-2-deoxy-D-mannono-1,4-lactone as a ligand appears to be very advantageous with regard to yield and purification. (Also, this column could be used repeatedly without loss of capacity, whereas the other two column-ligand combinations rapidly lost their capacity to retain enzyme.)

In all cases it can be seen that a constant ratio of glucosaminidase to galactosaminidase activity was obtained. This indicates once again that the activities belong to the same molecular species, and that the enzyme appears unable to distinguish the configuration at C-4 of the substrate. Kanfer et al. [13,18] have shown that β-glucosidase and β-galactosidase can be separated on a Sepharose column to which D-gluconolactone or D-galactonolactone had been coupled.

The hexosaminidase obtained by us after affinity chromatography (irrespective of which ligand-column had been used) showed two bands by acrylamide gel electrophoresis, one of which had enzyme activity.

We do not know why the 2-acetamido-2-deoxy-D-mannono-1,4-lactone, when coupled to sepharose afforded the highest degree of purification of the *N*-acetyl β-D-hexosaminidase. It may be that a mediocre inhibitor is better as an affinity-ligand because the enzyme will not bind to the column too strongly in that case. 2-Acetamido-2-deoxy-D-glucono-1,4-lactone, and the corresponding galactonolactone, have inhibition constants that are thousands of times higher than the one for 2-acetamido-2-deoxy-D-mannono-1,4-lactone [6].

Acknowledgements

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Table 1
Purification of 2-acetamido-2-deoxy-β-D-hexosaminidase

Lactone coupled to benzidine Sepharose 4B	Purification factor*	Ratio**	Yield*** %
2-Acetamido-2-deoxy-D-glucono-1,4-lactone	140	6.4	87
2-Acetamido-2-deoxy-D-galactono-1,4-lactone	120	6.5	82
2-Acetamido-2-deoxy-D-mannono-1,4-lactone	310	6.6	92

* Calculated from specific activities (munits/A_{280}) before and after column passage.
** The activity ratio is reported as the β-acetylglucosaminidase activity/β-acetylgalactosaminidase activity assayed by using the corresponding *p*-nitrophenyl glycosides.
*** The yields are reported as percentages of original enzyme activity applied to the column.

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