PURIFICATION OF *BACILLUS PUMILUS* β-D-XYLOSIDASE BY AFFINITY CHROMATOGRAPHY

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Received 23 October 1970

1. Introduction

We recently described the induction of a highly specific β-D-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.21) in a *Bacillus pumilus* strain [1]. However, the purification method yielded a non-homogeneous preparation, as judged by disc-gel electrophoresis. The present note deals with a very efficient purification procedure, based on affinity chromatography on *p*-aminobenzyl-1-thio-β-D-xylopyranoside coupled, as inhibitor-ligand, to a matrix of Sepharose 2B.

2. Materials and methods

The conditions for growth of the micro-organism and induction of the β-D-xylosidase were identical to those described previously [1]. The purification procedure, however, was modified and completed as described below.

Enzyme assays were performed with 4 mM *p*-nitrophenyl β-D-xylopyranoside as substrate, in 0.01 M phosphate (1 mM EDTA) buffer, pH 7.2 [1]. The fluorogenic substrate 4-methylumbelliferyl-β-D-xylopyranoside was used for visualization of enzyme active fractions after disc-gel electrophoresis. Protein concentrations were estimated at 280 nm [2].

p-Nitrobenzyl-1-thio-β-D-xylopyranoside triacetate was obtained by direct alkylation of the xylosyl mercaptan, according to Cerny et al. [3]. A mixture of 2,3,4-tri-*O*-acetylxylopyranosyl mercaptan (30 g; 0.03 mole) [4] in 90 ml acetone, *p*-nitrobenzyl bromide (20 g; 0.03 mole), and potassium carbonate (12.6 g) in 90 ml water, was agitated for 30 min at room temperature. An equal volume of water was added and the mixture extracted with chloroform. Evaporation of the chloroform and crystallisation of the residue from methanol yielded 38 g (78%) of *p*-nitrobenzyl-2,3,4-tri-*O*-acetyl 1-thio-β-D-xylopyranoside. The compound had a m.p. 88–90°C; $[\alpha]_D^{22}$ −88.9° (approx. 1 in $CHCl_3$); $[\alpha]_{436}^{22}$ −195.7° (approx. 1 in $CHCl_3$). Found: C 50.5; H 4.9. $C_{18}H_{21}NO_9S$ requires C 50.6; H 4.9%. The acetate was deacetylated [5], crystallized from ethanol and then from water, yielding *p*-nitrobenzyl 1-thio-β-D-xylopyranoside (yield 80%): m.p. 192.7 – 193.4°C; $[\alpha]_D^{22}$ −143.3° (approx. 0.4 in methanol); $[\alpha]_{436}^{22}$ −310.9°. Found: C 47.7; H 5.0. $C_{12}H_{15}NO_6S$ requires C 47.8; H 5.0%. This nitro derivate was hydrogenated [6] at atmospheric pressure, using Pd on $BaSO_4$ as catalyst. The product, *p*-aminobenzyl 1-thio-β-D-xylopyranoside (yield 74%), was crystallized from methanol and had m.p. 169.4–170.9°C; $[\alpha]_D^{22}$ −187.7° (approx. 0.4 in methanol); $[\alpha]_{436}^{22}$ −407.2°. Found: C 53.0; H 6.3. $C_{12}H_{17}NO_4S$ requires C 53.1; H 6.3%. The UV spectrum showed an absorption maximum at 246 nm.

2.1. *Coupling to Sepharose 2B*

A Sepharose 2B (Pharmacia) suspension (100 ml) was activated with 10 g CNBr in 100 ml water, according to Porath et al. [7]. After washing with cold 0.1 M $NaHCO_3$, the activated gel was suspended in 200 ml 0.5 M $NaHCO_3$ and 2 g *p*-aminobenzyl-1-thio-β-D-xylopyranoside was added. The mixture was rocked gently for 12 hr at room temperature, and then washed with 0.1 M $NaHCO_3$ and water until the absorption at 246 nm became negligible. When not in use, the absorbent was stored at 4°C, in water containing 0.1% sodium azide. In a similar manner

p-aminophenyl-1-thio-β-D-xylopyranoside was coupled to Sepharose 2B.

2.2. *Column chromatography*

The gel was packed into a column (3 × 19 cm), cooled at 4°C and washed with 0.02 M phosphate (1 mM EDTA) buffer, pH 6.8. The protein sample, containing the β-D-xylosidase, was dialysed against the same buffer and applied at the top of the column. After extensive washing (±50 ml/hr) with buffer, pH 6.8 (300 ml), successive elution with 2 M D-xylose and 0.1 M NaCl (both in the same buffer) was started.

Hydroxylapatite (Biorad) (column: 2.3 × 20 cm) was equilibrated with 0.02 M phosphate buffer pH 7.2 (1 mM EDTA) and cooled (4°C). Protein adsorbed was eluted with 0.1 M phosphate pH 7.2 (1 mM EDTA) in one step.

The protein contents of the column effluates were monitored at 257 nm (LKB Uvicord).

Disc-gel electrophoresis (7% acrylamide, pH 8.8) was performed as described previously [1]. However, gel and electrophoresis buffer contained, in addition, 1 mM EDTA.

3. Results and discussion

When a crude β-D-xylosidase preparation, obtained after a single ammonium sulfate fractionation (table 1, fraction III), was chromatographed on a Sepharose-*p*-aminobenzyl 1-thio-β-D-xylopyranoside column, the enzyme activity was retained, whereas the bulk of the protein material was eluted. 2 M D-xylose (K_i 26.2 mM) then specifically desorbed the enzyme (table 1, fraction IV). Some non-specifically bound, inactive protein, was eluted by 0.1 M NaCl.

In a control experiment, an enzyme sample was applied at the top of unsubstituted Sepharose. In this case, the β-D-xylosidase activity was eluted with the frontal protein peak. No detectable binding of the enzyme occurred when a column of Sepharose with coupled *p*-aminophenyl-1-thio-β-D-xylopyranoside was used. Thus, at least one bridging methylene group seems necessary.

Thus, by specific absorption and desorption on the Sepharose *p*-aminobenzyl-1-thio-β-D-xylopyranoside column, an almost integral separation of the glycosidase from the bulk of the protein material was achieved in one step.

Fraction IV was most effectively concentrated by adsorption on hydroxylapatite and freed from xylose by extensive washing with 0.02 M phosphate buffer pH 7.2 (1 mM EDTA). The enzyme was eluted with 0.1 M phosphate pH 7.2 (1 mM EDTA) in one step. An additional two-fold purification was achieved (fraction V).

For storage, fraction V was made 80% saturated with respect to ammonium sulfate and the precipitated

Table 1
Purification of β-D-xylosidase from *Bacillus pumilus* 12.

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (mU./mg)	Overall yield (%)	Overall degree of purification
I Crude extract (cell lysis and sonication)	553	332	8300	40	100	1.0
II Streptomycin	546	315	4500	70	95	1.8
III Ammonium sulfate (60–75%)	70	267	861	310	80	7.8
IV Affinity chromatography	429	243	76	3200	73	80
V Hydroxylapatite chromatography	54	211	34	6200	64	155

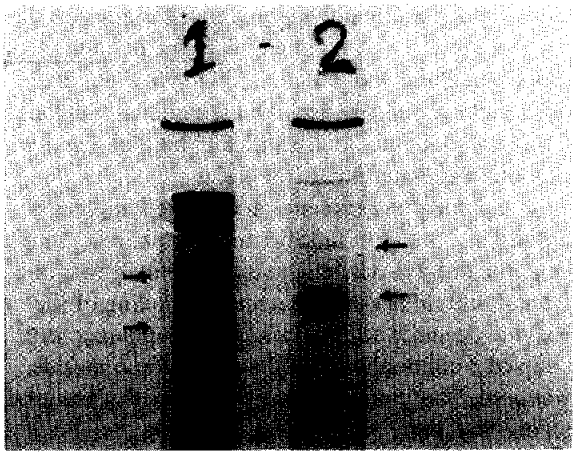

Fig. 1. Disc-gel electrophoresis of crude and pure β-D-xylosidase. Electrophoretic patterns of: (1) Fraction III (table 1); (2) Fraction V (table 1). Samples containing 30–50 μg protein were applied at the top of the columns. Electrophoresis was for 1.5 hr at 4 mA/tube with 7% gel. Arrows indicate enzyme active fractions (4-methylumbelliferyl-β-D-xylopyranoside).

enzyme was suspended in a minimum (3 ml) of 0.01 M tris-Cl buffer pH 7.2, 1 mM EDTA, saturated with ammonium sulfate.

On disc-gel electrophoresis, a second (weak) β-D-xylosidase band was detected both in the crude and purified samples (fig. 1). Work is now in progress to estimate the molecular weights and properties of these two β-D-xylosidase active fractions. It seems reasonable to suggest that they represent oligomeric forms of a single β-D-xylosidase.

To our knowledge, this report is the first concerned with the successfull purification of a glycosidase by affinity chromatography on a Sepharose matrix, coupled directly to a specific, synthetic inhibitor of low molecular weight, as outlined by Cuatrecasas et al. [8]. Different approaches, with other supporting matrices or ligand groups were reported [9, 10].

While the present manuscript was in preparation, the synthesis of several *p*-aminophenyl-1-thio-β-D-galactopyranoside derivatives coupled to Sepharose 4B was reported [11]. Only when attaching the inhibitor-ligand at considerable distance from the matrix was appreciable binding of a bacterial β-D-galactosidase noted.

Acknowledgements

We are indebted to Dr. F.G.Loontiens for helpful suggestions, to Mrs. Obreno-Cambier for valuable assistance and to Miss De Lat for performing the micro-analyses.

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