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Short Communication

Thiophilic interaction chromatography of sweet potato β-amylase

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ABSTRACT

The affinity of sweet potato β-amylase toward two kinds of thiophilic adsorbents [the so-called thiophilic or T-gel and 3-(2-pyridylsulphido)-2-hydroxypropylagarose or PyS-gel] was demonstrated. Enzyme adsorption on both gels was promoted by antichaotropic salts. Lower salt requirements and higher protein recoveries were observed for the PyS gel, even though its ligand concentration was half that of T-gel. For the former gel, the effectiveness series for sulphates was Na_2SO_4 > $(NH_4)_2SO_4$ > K_2SO_4 > $MgSO_4$. Based on the thiophilic character exhibited by the pure sweet potato β-amylase and the reversibility of the salt effects, a new method for its purification from crude extracts was developed.

INTRODUCTION

Sweet potato β-amylase (EC. 3.2.1.2) is a glucanomaltohydrolase, practical interest in which centres on its capacity to produce maltose syrups acting on liquefied starch from several sources, especially if it is combined with the use of pullulanase (debranching enzyme). The interest in this enzyme also resides in its application in continuous processes in immobilized form and the study of its mechanisms of action. For such purposes highly purified preparations are needed. There are many reports on purification procedures for sweet potato β-amylase, based on crystallization or acetone precipitation of the enzyme [1–3], affinity chromatography [4], glycogen precipitation [5,6], thymol–amylose complexation [7], ion exchange on DEAE-Sephadex A-50 [8,9] and hydrophobic interaction chromatography [10].

Porath *et al.* [11] introduced the use of a new class of group-specific adsorbents for protein chromatography, consisting of T-gel, with the general structure $M–OCH_2SO_2CH_2CH_2SCH_2CH_2OH$, and 2-thiopyridineagarose gel (PyS-gel) [12], with the structure $M–OCH_2CH(OH)CH_2SPy$, where M = matrix and Py = pyridyl. So far, their main applications have been in the purification of immunoglobulins from serum [11–13] and of monoclonal antibodies [14].

As demonstrated by Hutchens and Porath [15], the affinity of the T-gel is not restricted to immunoglobulins because, depending on the experimental conditions, other proteins also show thiophilic character. In this work, we found that pure sweet potato β-amylase also exhibits thiophilic behaviour and the quantitative effects of antichaotropic salts on its adsorption were evaluated. Based on these studies, a new procedure for its fast and efficient

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purification of the enzyme from unpigmented extracts by thiophilic interaction chromatography was developed.

EXPERIMENTAL

Materials

Sepharose 6B and DEAE-Sephadex A-50 were kindly provided by Pharmacia (Uppsala, Sweden). β-Mercaptoethanol, 2-mercaptopyridine and divinyl sulphone were purchased from Sigma (St. Louis, MO, USA). Soluble starch and 3,5-dinitrosalicylic acid were obtained from Carlo Erba (Milan, Italy). All other chemicals were of analytical-reagent grade.

Pure sweet potato β-amylase was obtained by hydrophobic interaction chromatography [10]. Its purity was tested by polyacrylamide gel electrophoresis.

Determination of enzyme activity and protein

Enzyme activity was assayed by the 3,5-dinitrosalicylic acid method [16] using 0.05 *M* sodium acetate (pH 4.8). Protein was determined spectrophotometrically at 280 nm, using an extinction coefficient ($E^{1\%}$, 280 nm) for β-amylase of 17.1 [17]; for the unpigmented extract the protein concentration was taken from the absorbance at 280 nm.

Enzyme unit, specific activity and enrichment ratio

One unit (U) of β-amylase activity is defined as the amount of enzyme that catalyses the production of 1 mg of maltose in 3 min at pH 4.8 under the assay conditions. The specific activity was defined as the number of enzyme units per milligram of protein. The enrichment ratio was expressed as the ratio of the specific activity after a given step and the initial step.

Synthesis of the thiophilic gels

T-gel was synthesized according to Porath *et al.* [11]. Essentially, 40 g of suction-dried Sepharose 6B were suspended in 40 ml of 0.5 *M* sodium carbonate solution (pH 11) and 2 ml of divinyl sulphone were added. The suspension was incubated with shaking for 18 h at room temperature and then thoroughly washed. The suction-dried activated gel was suspended in 40 ml of 0.1 *M* sodium hydrogencarbonate solution (pH 8.5) containing 2 ml of β-mercaptoethanol. The gel suspension was gently stirred for 20 h at room temperature and then thoroughly washed until free of β-mercaptoethanol. The ligand concentration was calculated to be 1312 μmol of ligand per gram of dried gel from sulphur elemental analysis of the gel before and after coupling with β-mercaptoethanol. PyS-gel was synthesized according to Porath and Oscarsson [12]. The ligand concentration calculated from sulphur and nitrogen elemental analysis was 657 μmol per gram of dried product.

Salt effects on the thiophilic adsorption of β-amylase

Aliquots of 0.5 g of suction-dried gels were incubated batchwise with gently mixing for 1 h at room temperature with 3.0 ml of sample (0.5 mg ml^{-1}) in the adsorption buffer *i.e.*, 0.1 *M* phosphate (pH 7.4) containing 0.5 *M* salts. Elution of the adsorbed protein was performed by salt deletion and quantified only in cases when more than 50% adsorption was obtained. The same procedure was followed to determine the optimum concentrations of ammonium sulphate and sodium sulphate to reach 90% enzyme adsorption on both gels.

Preparation of sweet potato unpigmented extract

A 200-g amount of peeled and sliced sweet potato (*Ipomea batata*) was blended in a Waring blender with 200 ml of water and then centrifuged for 30 min at 3000 *g* at 4°C. The supernatant was collected and diluted twofold with 0.1 *M* phosphate (pH 6.0)–0.6 *M* sodium sulphate. Pigments were removed by ion exchange on DEAE-Sephadex A-50 batchwise. Before application to PyS-gel, the pH was raised to 7.4.

Purification of β-amylase by thiophilic interaction chromatography

A 45-ml volume of an unpigmented extract was applied to a column of PyS-gel [6.0 ml of packed gel equilibrated in 0.1 *M* phosphate (pH 7.4)–0.3 *M* sodium sulphate]. The flow-rate was 25-ml h^{-1} and 5.0-ml fractions were collected. Desorption was performed by sodium sulphate deletion and with 30% ethylene glycol.

Disc polyacrylamide gel electrophoresis

This was carried out according to Orstein and Davis [18]. Protein staining was performed with 1%

amido black solution in 7% acetic acid. For enzym activity detection, gels run in parallel with stained gels were sliced into 3-mm pieces and incubated with 1 ml of activity buffer for 2 h. The enzyme activity was determined in the supernatants as described above.

RESULTS AND DISCUSSION

When pure β-amylase was applied to PyS-gel or T-gel in 0.1 *M* sodium phosphate (pH 7.4), only 10% of the protein applied was adsorbed. A possible way to promote protein adsorption on thiophilic gels was described by Porath *et al.* [11] for immunoglobulins using water-structuring salts. We investigated the effects of different sulphates at 0.5 *M* concentration on the enzyme adsorption on both gels (Table I).

These antichaotropic salts promoted adsorption on both gels to different extents; in both instances sodium sulphate was the most efficient. However, a difference in recovery of adsorbed protein by deletion of the salt was observed, *viz.*, 65% from T-gel and 90% from PyS-gel.

The percentage adsorption of β-amylase on both gels was quantified as a function of the final concentration of sodium sulphate in the range 0–0.5 *M* (Fig. 1). To reach 90% adsorption of the pure enzyme, the T-gel required 0.5 *M* and the PyS-gel 0.3 *M* sodium sulphate. The same experiments were performed with ammonium sulphate owing to its versatility and wide use in protein fractionation (Fig. 2). Higher ammonium sulphate concentrations were required to reach similar protein adsorption levels on both gels.

TABLE I

SALT EFFECTS ON THE THIOPHILIC ADSORPTION OF β-AMYLASE

Salt	T-gel		PyS-gel	
	Protein bound (%)	Recovery (%)	Protein bound (%)	Recovery (%)
$(NH_4)_2SO_4$	25	–	57	78
$MgSO_4$	36	–	21	–
K_2SO_4	21	–	54	58
Na_2SO_4	89	65	94	90

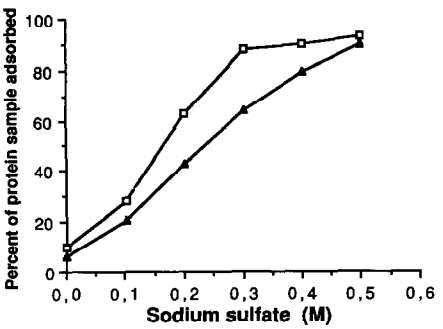

Fig. 1. Effect of sodium sulphate on the thiophilic adsorption of β-amylase on (□) PyS-gel and (▲) T-gel.

The lower salt requirements of Pys-gel justified its selection for β-amylase purification. The reversibility of the binding allowed quantitative desorption and made possible the application of this procedure to the purification of the enzyme starting with unpigmented extracts of sweet potatoes (Fig. 3).

Most of the other proteins present in the extract passed through the column, showing a lack of thiophilicity under the chromatographic conditions. The enzyme activity can be eluted as a sharp peak by deleting salt from the sample buffer, allowing its quantitative recovery in a very concentrated form. Other thiophilic proteins adsorbed on the gel were eluted under stronger conditions (30% ethylene glycol). The one-step purification procedure by thiophilic interaction chromatography for sweet potato β-amylase was highly reproducible and allowed an enrichment ratio of 29 to be obtained with a yield of 56.3% (Table II). The observed variations of these values in three independent experiments were *ca.* 10%.

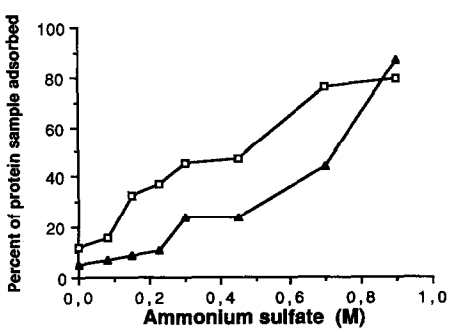

Fig. 2. Effect of ammonium sulphate on the thiophilic adsorption of β-amylase on (□) PyS-gel and (▲) T-gel.

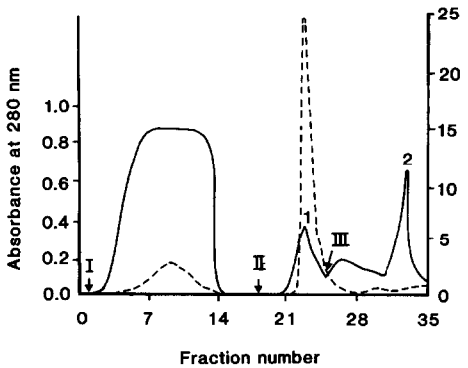

Fig. 3. Purification of β-amylase by thiophilic interaction chromatography on PyS-gel. I = sample application; II = desorption by sodium sulphate deletion; III = desorption with 30% ethylene glycol. Solid line, protein; dashed line, activity.

Electrophoretic analysis (Fig. 4) demonstrated the presence of only one band which was coincident with enzyme activity.

In conclusion, the thiophilic character of sweet potato β-amylase has been demonstrated. Enzyme adsorption on both types of gels was promoted by antichaotropic salts. As reported for immunoglobulins [13], sodium sulphate was the most effective of the four sulphates studied; ammonium, potassium and magnesium sulphates were much less effective in promoting adsorption of the enzyme on both thiophilic gels. Lower salt requirements (*e.g.*, 0.3 *M* sodium sulphate) and higher protein recoveries (*e.g.*, 90% of adsorbed enzyme) were observed for the PyS-gel, thus justifying its choice for the purification of the enzyme from crude extracts. In spite of the fact that the ligand content of PyS-gel was only half that of the T-gel, better binding properties

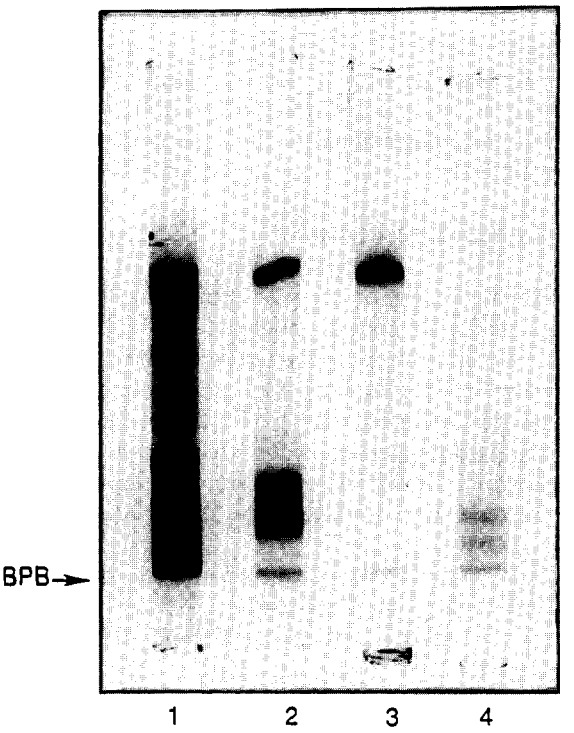

Fig. 4. Polyacrylamide gel electrophoresis (7.5% acrylamide, pH 8.3) at various stages in the purification of β-amylase: crude extract (lane 1), unpigmented extract to be applied to the column (lane 2), peaks 1 and 2 from Fig. 3 (lanes 3 and 4, respectively). BPB = bromophenol blue tracking dye.

toward β-amylase were exhibited by the former adsorbent. The high capacity and the possibility of reusing the same bed several times after regeneration make thiophilic interaction chromatography an extremely useful technique for enzyme purification.

TABLE II

SUMMARY OF β-AMYLASE PURIFICATION PROCEDURE

Fraction	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Enrichment ratio
Unpigmented extract	45	222	51	4.3	100	–
Active peak	5	125	1	125	56.3	29

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