Purification of α-Amylase Isoenzymes from *Scyatalidium thermophilum* on a Fluidized Bed of Alginate Beads Followed by Concanavalin A–Agarose Column Chromatography

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An α-amylase has been purified from the thermophilic fungus *Scyatalidium thermophilum*. A ninefold purification was achieved in a single step using fluidized bed chromatography wherein alginate was used as the affinity matrix. There are at least two isoenzymes as shown by concanavalin A (Con A)–agarose column chromatography. The isoenzyme binding to Con A is stable for at least 3 h at 80°C in the presence of calcium ions. The isoenzymes have similar molecular weights of around 45,000 Da as shown by SDS-PAGE analysis. The isoenzymes differ only slightly in their pH optima and temperature optima but the isoenzyme binding to Con A–agarose has slightly higher thermal stability.

**Key Words:** α-amylase; fluidized bed chromatography; alginate beads; *S. thermophilum*; thermostable enzymes.

α-Amylases constitute an important class of enzymes which find many biotechnological applications in processes which involve, for example, degradation of starch (1–5) and determination of soluble and insoluble dietary fiber in rice and wheat bran (6). Such applications include baking (7), brewing (8), detergents (9), and desizing (in textile industries). The potential advantage of using thermostable enzymes and organisms in the biotechnology industry has been reviewed earlier (2,10–14).

Most of the purification protocols reported for α-amylases from thermophilic sources and from mesophiles are multistep processes (15,16). Lately, however, the trend has been to cut down production costs in downstream processing by introducing affinity interactions right at the beginning of the purification protocol. Expanded bed affinity chromatography has emerged as a fairly powerful tool in large-scale purification of proteins (17–21). Recently, we have reported the affinity of amylases (from various sources) for alginate (22,23).

In this paper, we report the isolation and purification of two isoenzymes of α-amylase from a thermophilic fungus, *Scyatalidium thermophilum*, using a fluidized bed of alginate beads and a packed bed of concanavalin A–agarose as the affinity matrices.

**MATERIALS AND METHODS**

Sodium alginate (catalog no. A-2158, composed predominantly of mannuronic acid residues) and Con A–agarose† were purchased from Sigma Chemical Co. (St. Louis, MO). Protanal LF 10/60 (from brown seaweed, having high guluronic acid content (65–75%)) was a product of Protan A/S and was a gift from Prof. Bo Mattiasson (Lund University, Sweden). Soluble starch was a product of E. Merck. All other chemicals were of analytical grade.

**Estimation of Enzyme Activities and Amount of Protein**

The activity of α-amylase was estimated using starch as the substrate (24). The enzyme and the substrate were preincubated separately at 45°C for 3–4 min. The enzyme (0.5 ml) was incubated with 0.5 ml of the
substrate (1%, w/v) at a particular pH at 45°C for 5 min. The reducing sugar generated was measured using di- nitrosalicylic acid (25). One enzyme unit is defined as the amount of enzyme which liberates 1 µmol of reducing sugar (calculated as maltose) per minute at 45°C.

Protein was estimated by the dye-binding method, using bovine serum albumin as the standard protein (26).

**Determination of pH Optima**

The activities of the crude enzyme and the purified isoenzymes were assayed at different pH values in the range of 3.5–8.5.

**Determination of Temperature Optima**

The enzyme assay was carried out at pH 7.0 as above but the temperature of the assay was varied between 20 and 85°C.

**Polyacrylamide Gel Electrophoresis**

SDS-PAGE of the protein samples using 12% gel was performed according to Hames (27) on a Genei gel electrophoresis unit (Bangalore Genei Pvt. Ltd., Bangalore, India) with standard molecular weight markers (Amersham Pharmacia, Hong Kong).

**Culture**

*S. thermophilum* StB3 represents an isolate recovered from mushroom compost; it promotes the growth of *Agaricus bisporus* (28). The fungus was maintained on YpsSs agar at 4°C (29).

**Culture Conditions**

Aliquots (400 ml) of semisynthetic medium (soluble starch, 20 g; l-asparagine, 4.0 g; KH₂PO₄, 1.5 g; KH₂PO₄, 1.0 g; MgSO₄ · 7H₂O, 0.5 g; MnSO₄ · 4H₂O, 1.6 g; trace element solution, 0.01% (v/v) [trace element stock (mg ml⁻¹): FeSO₄ · 7H₂O, 5.0; MnSO₄ · 4H₂O, 1.6; ZnSO₄ · 7H₂O, 1.4; CoCl₂ · 6H₂O, 2.0]) were dispensed in 2000-ml Erlenmeyer flasks and the pH adjusted to 6.0. Flasks were autoclaved at 121°C and 15 psi for 20 min. Flasks were inoculated with a spore suspension (3 × 10⁵ spores) from a 4-day-old culture and incubated at 45°C on a rotary shaker (140 rpm) for 96 h.

**Preparation of Crude Enzyme Extract**

Culture broth was filtered through several layers of cheesecloth and stabilizers (Na₂EDTA, 0.001 M; PMSF, 0.001 M) were added to discourage proteolysis. The filtrate was stored at 4°C.

**Preparation of Alginate Beads**

Alginate beads were prepared from two different kinds of sodium alginate (high and low mannanuronic acid content) by a procedure outlined by Somers et al. (30). Beads were formed by dropping 50 ml of 2% alginate solution through a syringe into a 100-ml 0.1 M CaCl₂ solution. The beads (obtained from alginate with low mannanuronic acid) were kept for 2 h in CaCl₂ solution for hardening, washed with 0.006 M CaCl₂, and stored in a 0.006 M CaCl₂ solution at 4°C. For beads obtained from alginate with high mannanuronic acid content, the concentration of CaCl₂ used for hardening the beads was 0.2 M. These beads were also stored in 0.006 M CaCl₂ at 4°C.

**Optimization of Binding of Enzyme to Alginate Beads in the Batch Mode**

Three milliliters of the (appropriately diluted) crude extract each (containing 46 U of amylase activity) was equilibrated with 3 ml of both types of alginate beads

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**FIG. 1.** Optimization of conditions for the assay of amylase. (a) Determination of pH optimum of the enzyme: the pH optimum of the crude enzyme was measured by taking two different buffers for two different ranges, viz., 0.05 M acetate buffer (pH 3.5–6.5) (x) and 0.05 M Tris–acetate (pH 6.5–8.5) (○). Activity unit for the enzyme is defined in the text. The enzyme activity at pH 7.0 was taken as 100%. (b) Determination of optimum temperature: 0.5 ml of the enzyme was incubated with 0.5 ml of the substrate (0.05 M Tris–acetate, pH 7.0) at different temperatures for 5 min.
(high mannanuronic acid and high guluronic acid content) at three different pH values (0.05 M acetate buffer containing 0.006 M CaCl₂, pH 5.2, 0.05 M acetate buffer containing 0.006 M CaCl₂, pH 6.0, and 0.05 M Tris–acetate buffer containing 0.006 M CaCl₂, pH 7.0) for 1 h at 25°C. The bound activity was calculated by subtracting the activity remaining in the supernatant after incubation for 1 h from the initially added enzyme activity.

**Amylase Adsorption Isotherm**

Different aliquots of the crude extract containing α-amylase activity were made up to 2 ml with 0.05 M Tris–acetate buffer + 0.006 M CaCl₂, pH 7.0, added to 2 ml of beads (obtained from alginate with high mannanuronic acid content) and equilibration was carried out overnight at 25°C. Aliquots were removed and the amylase activity in the supernatant was measured to calculate bound amylase activity per milliliter of the adsorbent beads.

**Fluidization of the Settled Bed**

The equilibration buffer (0.05 M Tris–acetate buffer containing 0.006 M CaCl₂, pH 7.0) was pumped through a bed of adsorbent alginate beads (high mannanuronic acid content, bed volume 8.5 ml) packed in a 1.0 cm × 20.0 cm glass column (Supelco). The column volume was adjusted with the help of a polytetrafluoroethylene flow adapter fitted with a 20-mm polyethylene bed support. The flow to the bottom of the column was controlled using a peristaltic pump (Alitea AB, Model U4-MID). The sedimented adsorbent bed had a height of 11.0 cm. The liquid flow rate was first adjusted to calibrate the pump readings. The fluidization of the bed was then studied as a function of the liquid flow rate. Once the maximum bed height (as allowed by the column length) had been attained, the flow rate was decreased in steps and the bed height was measured at each flow rate.

**Fluidized Bed Mode**

Equilibration of the column was carried out as described above. Fifteen milliliters of the crude extract containing the enzyme was loaded into the column. The column was then washed with the equilibration buffer. Elution was carried out in the packed bed mode after switching off the pump and lowering the flow adapter to just above the settled bed. Ten milliliters of 1 M maltose (in 0.05 M Tris–acetate buffer containing 0.006 M CaCl₂, pH 7.0) was loaded into the column and kept for 4 h in a cold room. The eluate was dialyzed against the same buffer to remove maltose and amylase activity was determined (abbreviated as EI).

**Purification on Con A-Agarose Column**

The eluate from the above column was loaded into a Con A–agarose column (0.5 × 3 cm). The column was washed with 0.01 M phosphate buffer, pH 6.9, containing 0.001 M MgCl₂, 0.001 M MnCl₂, and 0.001 M CaCl₂. The activity in the unbound fraction is abbreviated as
thermal stabilities of the EII and EIII fractions were determined at 80°C in the presence of 0.006 M CaCl₂.

RESULTS AND DISCUSSION

The crude enzyme extract after filtration was assayed for α-amylase activity as described below.

Optimization of Assay Conditions

The assay conditions for the enzyme were optimized by determination of the pH optimum (Fig. 1a) and temperature optimum (Fig. 1b). The enzyme has a fairly broad pH optimum around 5.5–7.5. This is similar to the pH optima for other α-amylases (31–34). The temperature optimum was around 45°C. The enzyme was assayed at pH 7.0 using 0.05 M Tris–acetate buffer at 45°C.

Binding of α-Amylase to Alginate Beads

Recently, it was reported that α-amylases can be purified by using alginate as affinity material (22,23). It was also observed that while some α-amylases bind more efficiently to alginate consisting of primarily mannuronic acid residues, others show greater affinity for alginate with high guluronic acid content (35). Figure 2 shows that α-amylase from S. thermophilum binds more efficiently at pH 7.0 and with the alginate with high mannuronic acid content. The beads pre-
Purification of Fungal α-Amylase on a Fluidized Bed of Alginate Beads

<table>
<thead>
<tr>
<th>Steps</th>
<th>Volume (ml)</th>
<th>Activity (U)</th>
<th>Protein (mg)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield of activity (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude fungal extract</td>
<td>15.0</td>
<td>345.6</td>
<td>1.2</td>
<td>260.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Wash</td>
<td>80.0</td>
<td>45.8</td>
<td>0.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Eluate (ElI)</td>
<td>10.0</td>
<td>245.0</td>
<td>0.1</td>
<td>2450.3</td>
<td>80.2</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Note: The enzyme from the crude filtrate was loaded on the column with settled bed height of 11 cm. The column was fluidized by pumping in 0.05 M Tris–acetate buffer containing 0.006 M CaCl₂, pH 7.0, until the bed attained a height 1.46 times the settled bed height. The rest of the experimental details are described in the text.

Prepared from alginate with high mannuronic acid content were thus used for further studies.

Adsorption Isotherm

Quantitative assessment of the results was done by fitting the data to a Langmuir isotherm of the form (36)

\[ q^* = \frac{q_m c^*}{K_d + c^*} \]

\( c^* \) is the equilibrium liquid concentration in U ml⁻¹, and \( q^* \) is the capacity of the matrix (U ml⁻¹) at a particular enzyme load. \( K_d \) is the dissociation constant and \( q_m \) is the maximum capacity of the matrix. The adsorption isotherm of the fungal amylase on beads (from alginate of high mannanuronic acid content) (Fig. 3) was found to fit the Langmuir adsorption pattern (36).

The maximum capacity \( (q_m) \) of the alginate beads for the fungal amylase was found to be 14.7 U ml⁻¹ and the dissociation constant \( (K_d) \) equaled 0.66 U ml⁻¹. The maximum capacity of the alginate beads for the enzyme is in the same range as found for amylases from other sources (35) but these alginate beads display stronger affinity for this enzyme as compared to other amylases.

Fluidized Bed Chromatography

The amylase was purified on a fluidized bed of alginate beads (Fig. 4). The enzyme could be purified about ninefold, with 80% recovery of enzyme activity (Table 1). SDS–PAGE analysis revealed significant purification (Fig. 5). The minimum molecular weight appears to be around 45 kDa, which is similar to values reported for many plant enzymes (37).

Thermal Stability of the Purified Enzyme

The thermal stability of the purified enzyme was determined at 80°C with and without the addition of Ca²⁺ ions (Fig. 6). In both cases, the enzyme lost two-thirds of its activity almost immediately at this temperature. However, in the presence of Ca²⁺, the remaining activity was quite stable up to 3 h. Assuming first-order thermoinactivation kinetics (38), the half-life \( (t_{1/2}) \) for this surviving activity in the presence of Ca²⁺ works out to be 38 h. This thermostabilization by Ca²⁺ is in agreement with results reported for other amylases (37). This behavior suggested that there may be two isoenzymes with similar molecular weights but with different thermostabilities.

Separation of Isoenzymes on Concanavalin A–Agarose Column

As fungal amylases are known to be glycoproteins (39), the purified enzyme was passed through a Con A–agarose column. About one-third of the α-amylase activity bound to the column and it could be quantitatively eluted (ElII) with 0.01 M α-methyl manno-pyranoside. The specific activity of the fraction ElII was 2530 U mg⁻¹ whereas that of ElI (the fraction which did not bind to Con A–agarose) was 2389 U mg⁻¹. The thermal stability of the two fractions, viz., eluted from the Con A–agarose column (ElIII) and unbound to the column (ElI), was reexamined at 80°C in the presence of Ca²⁺ ions. The α-amylase activity of the ElIII fraction

![FIG. 5. SDS–PAGE of the purified enzyme. Lane 1, fraction ElI (5 μg) (eluate from column of alginate beads); lane 2, fraction ElII (6 μg) (part of ElI which did not bind to Con A–agarose); lane 3, fraction ElIII (5 μg) (part of ElI which bound to Con A–agarose and was eluted with α-methyl mannoside); lane 4, crude extract (10 μg); lane M, marker proteins. The gel was stained with Coomasie brilliant blue R-250 for 45 min and then destained in 40% methanol and 10% acetic acid.](image)
remained completely intact after exposure (up to 3 h) at 80°C. On the other hand, EII became inactive immediately on exposure at 80°C. The results clearly indicate that we are dealing with (at least) two isoenzymes of α-amylases, presumably differing in their carbohydrate content. The isoenzyme with carbohydrate residues specific for Con A has higher thermal stability. This is as per the delineated role of carbohydrate residues in the stability of glycoproteins/glycoenzymes (40).

**Properties of Isoenzymes**

The pH optimum of the EII fraction was in the range of 6.0–7.5 and that of the EIII fraction was slightly broader and in the range of 5.5–7.5 (data not shown). The temperature optima of the EII and EIII fractions were 45 and 50°C, respectively (data not shown). Both EII and EIII showed identical mobilities on SDS-PAGE as EII (Fig. 5). Thus, the two isoenzymes EII and EIII (components of EII) have identical molecular weights.

The α-amylase isoenzymes isolated and described above have an important biochemical role, as described previously. Also, thermophilic fungi play an important role in compost preparation. *S. thermophilum* is abundant in mushroom compost and its population is shown to be positively correlated with mushroom yield (28). Thus, purification and characterization of such enzymes from this organism will help in developing the molecular-level picture of compost utilization.

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