PURIFICATION AND IMMOBILIZATION OF *SCYTALIDIUM* SP. α -AMYLASE AND ITS GENERAL PROPERTIES

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An α -amylase of *Scytalidium* sp. was produced in shake flask cultures. The enzyme was purified to a homogeneous state on polyacrylamide gel electrophoresis by acetone precipitation, DEAE-cellulose column chromatography and Sephadex G-75 gel filtration. The molecular weight of the enzyme was about 87,000 dalton. Metal ions such as Fe^{2+} , Na^+ and Cu^{2+} activated the enzyme activity while Hg^{2+} and Zn^{2+} were strongly inhibitory. The α -amylase was immobilized on DEAE-cellulose by adsorption. The optimum pH of the immobilized enzyme was 6.0, as compared with 8.0 for the free enzyme. The immobilized enzyme was optimally active at 50°C and retained 50% activity at 100°C (30 min) as against 40°C and 40% (100°C, 30 min) for the free enzyme.

α -Amylase (EC 3.2.1.1) hydrolyzes the α -1,4-glucan bonds in starch, glycogen, and related α -1,4-glucans (16). Studies on fungal amylases have concentrated mostly on the genera *Aspergillus* and *Rhizopus*, probably because of their ubiquitous distribution and non-fastidious nutritional requirements. The enzymes of *Aspergillus niger* and *Aspergillus oryzae* have been commercialized (3).

Scytalidium is a saprophytic soil mold whose type species *Scytalidium hyalinum* has been reported (4) to cause opportunistic superficial infections of the skin and nail.

Immobilization of enzymes has made it possible for industrial applications of enzymes of non-GRAS (Generally Recognized As Safe) organisms (7,8). In this paper, the purification, immobilization and some properties of α -amylase of *Scytalidium* sp. are described.

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MATERIALS AND METHODS

Organism. A soil isolate of *Scytalidium* sp. from the authors' was used. Stock cultures were maintained on glucose yeast extract mineral salts agar (15) slants and stored at 4°C.

Culture conditions and amylase production. Time course of amylase production was studied in 250 ml Erlenmeyer flasks each containing 50 ml of culture medium of the following composition (g/l): KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; NaCl, 0.5; NaNO_3 , 2; bacteriological peptone, 30; soluble starch, 40 (pH 5.5). Fermentation was carried out at 28°C in an orbital incubator (160 rpm) for 6 days during which pH, biomass (estimated as dry weight of mycelium), starch disappearance and amylase production were monitored daily.

For enzyme production, four 250 ml Erlenmeyer flasks each containing 50 ml of fermentation medium of a similar composition to the medium described above were used, except that bean (cow pea) meal (30 g/l) replaced bacteriological peptone. Each flask was inoculated with three agar plugs (1 cm diameter) of 48 h culture of *Scytalidium* sp. The flasks were incubated in an orbital shaker (160 rpm, 28°C) for 96 h. Mycelia and suspended solids were removed by centrifugation ($5,000 \times g$, 10 min, 4°C) and the clear supernatant was used as enzyme source.

Assay of amylase activity. Amylase activity was measured in 1 ml containing 0.5 ml of 1% (w/v) soluble starch (Hopkin and Williams Ltd.) in 20 mM phosphate buffer (pH 7.0) and 0.5 ml of enzyme solution. After 10 min at 37°C, reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNS) method (12). The activity of one unit (U) of amylase is defined as that amount of enzyme which is able to liberate 1 mg of reducing sugar as a glucose standard per minute under the assay conditions.

The pattern of release of products of starch hydrolysis, i.e., the slow release of reducing equivalents and concomitant fast reduction in iodine staining value of starch, strongly indicates α -amylolysis.

Amylase purification. All steps of purification were carried out at 4°C unless otherwise stated. Clear culture supernatant (150 ml) was subjected to cold (-10°C) acetone precipitation. The precipitates were recovered by centrifugation ($3,000 \times g$, 10 min) and suspended in 20 mM phosphate buffer (pH 7.0). The enzyme solution (80 ml, 6.4 mg protein) was applied on a pre-equilibrated DEAE-cellulose DE 32 column (2×12.5 cm). Proteins were eluted with 20 mM phosphate buffer (pH 7.0) and a linear gradient of NaCl (0–0.5 M) at a flow rate of 20 ml/h (5 ml fractions). Fractions eluting between 0.15–0.19 M NaCl and with high amylase activity were pooled, concentrated by acetone precipitation and the enzyme solution loaded onto a Sephadex G-75 column (2×45 cm) equilibrated with 20 mM phosphate buffer (pH 7.0). Fractions of 5 ml per tube were collected and amylase-active ones were pooled and concentrated in 25 ml volume in 20 mM (pH 7.0) as described above, and stored frozen.

Protein measurement. Protein was estimated using the method of Lowry (11) and by measuring the absorbance at 280 nm.

Homogeneity test. The homogeneity of the purified enzyme was tested by disc polyacrylamide gel electrophoresis (5). For staining of gel, Coomassie brilliant blue (Sigma Chemical Co.) was used.

Estimation of relative molecular weight. The method of Andrews (1) was used on Sephadex G-75 column (2×45 cm) equilibrated with 20 mM phosphate buffer. The void volume was determined with blue dextran (see legend to Fig. 3 for marker proteins).

Thermostability test. The method described by Kochhar and Dua (9) was adopted. The purified enzyme was placed in thin-walled test tubes and incubated at different temperatures for 30 min. Thereafter, the tubes were promptly chilled in ice and the remaining enzyme activity assayed as earlier described.

Enzyme immobilization. α -Amylase was immobilized on DEAE-cellulose matrix as described by Krakowiak and Obeta (10). Ten grams of carrier was suspended in 1 M NaOH (150 ml, 30 min), washed with distilled water until the pH of the carrier was neutral. The carrier was then suspended in 0.5 M NaCl (150 ml, 30 min), washed as earlier described until the pH was 7.0. Thereafter, it was washed with 150 ml of 1 M NaOH and distilled water followed by 20 mM (200 ml) phosphate buffer (pH 4.5) and thereafter packed in a glass column (2×20 cm). The column was further equilibrated with 300 ml of 20 mM phosphate buffer (pH 4.5), and dialyzed α -amylase (10 ml) was added into the column. This was then washed with 20 mM phosphate buffer (pH 4.5) until the eluate was free from amylolytic activity. The column was then unpacked and the carrier was stored at 4°C.

Assay of immobilized α -amylase. The method of Ohba et al. (14) was adopted. The reaction mixture consisted of 5 mg of immobilized enzyme in 3 ml of 20 mM phosphate buffer (pH 7.0), and 1 ml of 1% soluble starch. This was then incubated at 37°C (10 min) with stirring. After color development with DNS reagent, the immobilized enzyme was removed by centrifugation ($2,000 \times g$, 10 min) before measurement of the absorbance at 540 nm. An amylase unit (U) is defined as the amount of immobilized enzyme which produces 1 mg of reducing sugar as glucose per min under the assay conditions.

RESULTS

Pattern of amylase production by Scytalidium

The time course of amylase synthesis by *Scytalidium* sp. is shown in Fig. 1. Amylase production started during the exponential growth phase of the fungus, attaining a maximum after 96 h and corresponding with the culture pH of 4.4. About 60% of the starch in the medium was utilized at the point of maximum enzyme production.

Fig. 1. Time course of α -amylase synthesis by *Scytalidium* sp. ○: α -amylase, ●: starch disappearance, □: biomass, ■: pH.

Table 1. Purification of α -amylase of *Scytalidium*.

Steps	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification
Supernatant from culture broth	150	8,509	29	293	100	1
Acetone precipitation	100	5,263	8.5	619	62	2.1
Ion-exchange chromatography on DEAE-cellulose	75	2,105	2.25	936	25	3.2
Gel filtration on Sephadex G-75	25	467.8	0.25	1,871.2	5.5	6.4

Enzyme purification and homogeneity

α -Amylase was purified (6.4 fold, specific activity 1,871.2 U/mg protein) by acetone precipitation, ion-exchange chromatography and gel filtration. The purification summary is outlined in Table 1. The purified α -amylase appeared homogeneous based on two criteria: protein and activity profiles coincided in the gel filtration (figure not shown) and a single protein band was obtained when the purified enzyme was subjected to electrophoresis (Fig. 2).

Relative molecular weight

The relative molecular weight of the amylase was estimated to be 87,000 dalton (Fig. 3).

Fig. 2. Gel electrophoresis of α -amylase of *Scytalidium* sp. Electrophoresis was carried out using the following conditions: buffer, 3 mM Tris-glycine, pH 8.3; current, 5 mA per tube; duration, till tracking dye (0.001% w/v bromophenol blue) emerged.

Fig. 3. Estimation of the relative molecular weight of α -amylase of *Scytalidium* sp. Standard proteins used were: (●) myoglobin; (×) trypsin; (▲) bovine serum albumin; (■) lactate dehydrogenase; (□) pyruvate kinase. The open circle denotes α -amylase.

Physico-chemical properties

The optimum temperature for the activity of the unbound amylase was 40°C but it retained 40% of its original activity at 100°C (30 min) (Fig. 4A). The immobilized enzyme was optimally active at 50°C and retained more than 50% of its activity at 100°C for 30 min (Fig. 4B).

Figure 5A and 5B show the pH activity and stability profiles of the unbound and bound amylase, respectively. The free amylase was optimally active at pH 8 while the bound enzyme was most active at pH 6. However, both forms of the

Fig. 4. Effect of temperature on the activity (×) and stability (O) of: (A) free α -amylase, (B) immobilized α -amylase.

enzyme were stable between pH 6 and 8 (37°C, 24 h).

Catalytic properties

The effects of various metal ions on amylase activity were examined (Table 2). Fe^{2+} enhanced amylase activity by 33.7% followed by Na^+ (3.7%) and Cu^{2+} (1.2%). All the other metal ions tested were inhibitory with maximum inhibition

Table 2. Effect of metal ions on α -amylase activity.

Metal salt (5 mM)	Relative activity (%)
None	100
FeSO ₄	133.7
Na ₂ S ₂ O ₃	103.7
CuSO ₄	101.2
MgSO ₄	75.6
KNO ₃	73.1
BaCl ₂	29.3
CaCl ₂	28.7
AgNO ₃	28.1
ZnCl ₂	25.0
HgCl ₂	8.7

The reaction mixture contained soluble starch (1%) in 20 mM phosphate buffer (pH 7.0), metal salt (5 mM), and the enzyme in a total volume of 1 ml.

Table 3. Relative rates of hydrolysis of starch by free and immobilized α -amylase.

Starch (%)	Relative rate of hydrolysis (%)	
	Free amylase	Immobilized amylase
Potato (soluble)	100	100
Sorghum	90.7	92.1
Maize	86.7	87.3
Cassava	84.0	85.7
Cocoyam	70.0	72.0

Table 4. K_m values of free and immobilized amylase of *Scytalidium* sp.

Substrate	K_m (mg/ml)	
	Free enzyme	Immobilized enzyme
Soluble starch (potato)	2.3	0.83
Amylopectin	5	1.25
Glycogen	10	2

(91.3%) achieved with Hg²⁺.

Comparison of the relative rates of starch hydrolysis by free and immobilized amylase is shown in Table 3. Both forms of the enzyme displayed high catalytic activity with the starches. Soluble starch was hydrolyzed fastest while cocoyam starch was the least hydrolyzed. Slightly higher rates of starch hydrolysis were, however, obtained with immobilized α -amylase over values obtained with the free enzyme.

Paper chromatography of digests (2 h) of amylaceous polysaccharides demonstrated the production of glucose, maltose and malto-oligosaccharides from potato amylopectin, rabbit liver glycogen and soluble starch (figure not shown).

The Michaelis constants (K_m) of the free amylase were 3.3 mg/ml, 5 mg/ml and 10 mg/ml for soluble starch, amylopectin and glycogen, respectively. For the bound enzyme, the values were 0.83 mg/ml, 1.25 mg/ml and 2.0 mg/ml for soluble starch, amylopectin and glycogen, respectively (Table 4).

The pattern of release of reducing power and reduction in iodine-staining capacity of *Scytalidium* sp. α -amylase, sweet potato β -amylase (extracted in our

Fig. 5. Effect of pH on the activity ($\times$) and stability ($\circ$) of: (A) free α -amylase, (B) immobilized α -amylase. Buffers used were: 20 mM citratephosphate, pH 2-4; 20 mM sodium phosphate, pH 6-8; 20 mM NaH_2PO_4 -NaOH, pH 10-12.

Fig. 6. Reduction in iodine staining value and the release of reducing power from starch. ●: *Scytalidium* α -amylase, ○: *Bacillus subtilis* α -amylase, ⊙: sweet potato β -amylase. The reaction mixture consisting of 1% (w/v) starch solution (5 ml) in buffer and enzyme (5 ml) was incubated at 40°C. At intervals, samples (0.5 ml) were withdrawn and added to 2 ml of 0.1 M H₂SO₄. Iodine solution (5 ml, containing 0.05 g I₂/l and 0.5 g KI/l) was added and the absorbance measured at 600 nm against a water/iodine blank. Reducing power was measured with DNS reagent.

laboratory) and *Bacillus subtilis* α -amylase (Sigma Chemical Co.) is shown in Fig. 6. Both *Scytalidium* sp. α -amylase and *Bacillus subtilis* α -amylase rapidly reduced the iodine-staining capacity of soluble starch with slow release of reducing power. On the contrary, sweet potato β -amylase slowly reduced the iodine-staining capacity of starch with concomitant rapid release of reducing power.

DISCUSSION

The enzyme of *Scytalidium* sp. was classified as α -amylase based on its ability to rapidly reduce the iodine-staining capacity of soluble starch and the slow production of reducing equivalents (Fig. 6) when compared with authentic α -amylase (*Bacillus subtilis* α -amylase, Sigma Chemical Co.) and β -amylase (sweet potato β -amylase extracted in our laboratory). Starch hydrolysis by endo-attacking enzyme is accompanied by rapid reduction in the iodine-staining capacity but slow release of reducing power (13). The amylase produced glucose, maltose and malto-oligosaccharides from amylopectin, glycogen and soluble starch. These products further suggest endo-hydrolytic pattern of the amylase, and thus resembles the α -amylase of *Bacillus licheniformis* NCIB 6346 in which the products of amylolysis were glucose, maltose and malto-oligosaccharides.

α -Amylase production paralleled mycelial growth, reaching a peak at 96 h. Starch completely disappeared from the medium after 144 h although mycelial growth continued. This observation is similar to the pattern of α -amylase synthesis

by an *Aspergillus* sp. in which maximum enzyme production occurred at 96 h (17).

The purified α -amylase appeared as a single protein band on gel electrophoresis. Its relative molecular weight was calculated to be 87,000 dalton. This figure for the amylase is high when compared to 61,000 and 51,000 reported (2,6) for *Aspergillus niger* and *Aspergillus oryzae* α -amylase, respectively.

Immobilization of the enzyme on DEAE-cellulose improved the heat stability of the enzyme. The variation in temperature optimum and stability for both the free and bound enzymes is in consonance with the observation of Gutcho (8) that immobilized enzymes have a wider range of temperature activity and stability profiles. However, this observation might not be real since there is the tendency for a relatively higher local concentration of the immobilized enzyme in the reaction mixture thereby raising the enzyme stability and other properties (20).

Purified free α -amylase of *Scytalidium* sp. was unstable at higher temperatures, having retained only 40% of the original activity at 100°C.

Many workers have reported that α -amylases are activated and stabilized by Ca^{2+} but this is not true in our investigations. Probably Ca^{2+} does not contribute towards maintaining the active conformation of the α -amylase of *Scytalidium* sp. This observation was reported by Srivastava et al. (19) for the α -amylase of a thermophilic *Bacillus* sp. Also it is possible that the Ca^{2+} might bind to the phosphate in the buffer to form calcium phosphate which reduces the concentration of dissolved Ca^{2+} in the reaction mixture and thus the α -amylase activity (18).

The enzyme demonstrated highest affinity for soluble starch than glycogen and amylopectin as exemplified by their K_m values. However, lower K_m values were obtained with the bound enzyme.

Although the type species *Scytalidium hyalinum* can cause opportunistic skin and nail infections of man (4), immobilization of the α -amylase of *Scytalidium* sp. could enhance its possible use in industry.

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