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Purification and some properties of α-amylase from post-harvest *Pachyrhizus erosus* L. tuber

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Abstract

α-Amylase, a starch splitting enzyme, was purified to homogeneity from post-harvest *Pachyrhizus erosus* L. tuber by successive chromatography on DEAE- and CM-cellulose columns. Purification achieved was 110 fold from the crude extract with a yield of 22.8%. SDS-PAGE showed a molecular weight of 40 kDa for the enzyme. The enzyme is of α-type as it lost total activity in the presence of EDTA, a chelating agent. It is a glycoprotein that contains 2.6% sugar as estimated by the phenol-sulfuric acid method. The enzyme displayed optimum activity at pH 7.3 and 37 °C with an apparent K_m value of 0.29% for starch as substrate. The enzyme was strongly inhibited by Cu^{2+}, Fe^{2+} and Zn^{2+}, moderately by Li^{2+}, Hg^{+} and Cd^{2+} and slightly by Ag^{+}, Mg^{2+} and K^{+}. Calcium ion almost doubled the activity whereas Fe^{3+}, Mn^{2+} and Na^{+} enhanced it appreciably.

Keywords: *Pachyrhizus erosus* L. tuber; α-Amylase; Glycoprotein; Characterization

1. Introduction

α-Amylase, an important industrial enzyme, is used in starch liquefaction to produce glucose, fructose and maltose and also in brewing, baking, textile, paper, detergent and sugar industries (Crueger & Crueger, 1990). It has been purified from different tubers (Teotia, Khare, & Gupta, 2001) and from many other sources (Lee, Kim, & Ji, 1997; Lizotte, Henson, & Duke, 1990). Amylase activity in potato during different stages of maturation has been reported (Nielsen, Christensen, Bojko, & Marcussen, 1997).

Pachyrhizus erosus is commonly known by several names, such as Sakaloo or Kesor Aloo (Bengali), Jambohnc or Knolicnbohnc (German), Yam (English name). It is one of the most important crops in terms of food value and wide adaptability. An appreciable activity of amylase in post-harvest *P. erosus* tuber was observed in our study. We have also studied the nutritional value and anti-nutrient factors, if present in the tuber. The tuber could be consumed fresh as a supplement of crude fibre and as a good source of minerals and vitamins. In order to increase the consumer acceptance, the sweetness should also be increased to a certain level. This needs enhancement of amylase activity above the existing level. For the purpose, it is necessary to study the enzyme in terms of genetic engineering. To our knowledge data on the purification and characterization of *P. erosus* tuber amylase are not available. Our first approach was to purify the enzyme. This paper describes the purification and characterization of α-amylase from post-harvest *P. erosus* tuber.

2. Materials and methods

2.1. Materials

The study was conducted with *P. erosus*, a single variety harvested from a field of Bagha Mosque (longitude 88°45′–88°45′, latitude 24°05′–24°20′), Bagha, Rajshahi, Bangladesh.

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DEAE-cellulose and CM-cellulose were purchased from Sigma Chemical Co., USA. All other reagents used were of analytical grade.

2.2. Crude enzyme preparation

All the operations were performed at 4 °C unless otherwise specified. *P. erosus* tuber was peeled, sliced and ground to a paste in a mortar with pestle in (1 g/ml) precooled 50 mM phosphate buffer, pH 7.4 and finally homogenized by a tissue homogenizer. The suspension was then filtered through four layers of gauze. The filtrate was clarified further by centrifugation at 8000*g* for 15 min. The clear supernatant was concentrated to about 14% of the original volume by commercial sucrose. The supernatant was taken in a dialysis bag and sucrose crystals were kept around the dialysis bag so that water can diffuse out resulting in a decrease in volume of the supernatant. The concentrate was dialyzed against deionized water for 12 h and subsequently against 50 mM phosphate buffer (pH 7.4) for 12 h with four changes of buffer. The dialyzed sample was centrifuged at 8000*g* for 8 min and the supernatant was used as crude enzyme extract.

2.3. DEAE-cellulose chromatography

The crude enzyme extract was loaded on to a DEAE-cellulose (DE-52) column (2.1 × 24 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.4 at 4 °C. The column was thoroughly washed with the equilibration buffer until the UV absorbance of the column eluate returned to the base line. The bound protein was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled and purified further by CM-cellulose chromatography.

2.4. CM-cellulose chromatography

The active fractions obtained from DEAE-cellulose chromatography were concentrated by commercial sucrose (as described in Section 2.2) and dialyzed against 50 mM sodium phosphate buffer, pH 6.5 for 24 h. The dialyzed sample was centrifuged and the supernatant was loaded onto the CM-cellulose (CM-50) column (2.1 × 24 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.5. The column was washed with the same buffer until the absorbance of the column eluate returned to the base line. The bound protein was then eluted with a gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled for characterization.

2.5. Protein and enzyme assay

Protein was estimated by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as the standard. The protein content of the column eluate was monitored by measuring the absorbance at 280 nm. Amylase activity was assayed by the dinitrosalicylate (DNS) method of Miller (1959). Three sets of reaction mixtures (blank, control and sample), prepared for the assay of amylase activity, contained 2.5 ml of 1% starch solution in 0.1 M phosphate buffer, pH 6.5, and 1 ml of 1% NaCl. The contents in the tubes were mixed uniformly and incubated at 37 °C for 10 min. Then, 0.5 ml of enzyme was added to both the sample and control tubes, but immediately after addition of enzyme, 0.5 ml of 2 N NaOH was added to the control tube to stop the reaction. In the blank tube, 0.5 ml of distilled water was added in place of enzyme. Then the tubes were incubated at 37 °C for 15 min and 0.5 ml of 2 N NaOH added to the sample and blank tubes to stop the reaction. Then 0.5 ml of DNS reagent was added to each tube and it was vortexed. The tubes were heated in a boiling water bath for 5 min. After cooling to room temperature the absorbance was measured at 520 nm. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of maltose/min under standard assay conditions.

2.6. Electrophoresis

Purity and the molecular weight of the purified α-amylase were determined by native polyacrylamide gel electrophoresis (native PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively, using the Laemmli (1970) gel method on 10% polyacrylamide slab gel containing 1% SDS. Gels were stained with Coomassie Brilliant Blue. Standard proteins used for calibration were lysozyme (14 kDa), trypsin inhibitor (20 kDa), pepsin (36 kDa), egg albumin (45 kDa), and bovine serum albumin (67 kDa).

2.7. Kinetic constants

Kinetic parameters of the purified amylase for starch as substrate were determined at pH 6.7 and 37 °C. The values of Michaelis constants (K_m) and the maximum velocity (V_{max}) were determined from Lineweaver–Burk plots.

2.8. Glycoprotein test and sugar estimation

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric micro determination of sugars and their methyl derivatives, oligosaccharides and polysaccharides, as described by Dubois, Gilles, Hamilton, Rebers, and Smith (1956). The method was also applied for the detection and estimation of sugar in protein.

2.9. Optimum pH and temperature

The optimum pH for the amylase activity was determined by assaying the activity at different pH values, using the following buffers: 0.1 M acetate buffer (pH 3.0–5.5), 0.1 M phosphate buffer (pH 6.0–7.5) and 0.1 M Tris–HCl

buffer (pH 8.0–9.0). The optimum temperature for amylase activity was determined by assaying the enzyme at temperatures from 10 to 90 °C at pH 6.7.

2.10. pH and heat stability

Stability of the enzyme at different pH values was determined by measuring the residual activity after incubating the enzyme at pH 3.0–9.0 for 24 h at 37 °C. The assay conditions were otherwise similar to that given above. Heat stability was measured by incubating the enzyme at 30–80 °C for 30 min in 0.1 M phosphate buffer, pH 6.7. After heat treatment, the enzyme solution was cooled and the residual activity assayed under standard assay conditions.

2.11. Substrate specificity

To determine the substrate specificity of the enzyme, several raw starches, such as potato, maize, wheat and rice, soluble starch, oligosaccharides and polysaccharides, such as maltotetraose, amylose and amylopectin, were used as substrates during the assay. Reducing sugar released was assayed by the standard procedure (DNS), as described earlier.

2.12. Metal ion effect

The effects of various metal ions and EDTA on enzyme activity were determined by pre-incubating the enzyme (0.25–0.3 mg/ml) with 0.5 ml of individual reagent at specified concentrations for 30 min, followed by incubation with starch under the standard assay conditions and then assaying the enzyme activity. The activity assayed in the absence of metal ions or reagents was taken as 100%.

3. Results and discussion

3.1. Purification of amylase

Purification of *P. erosus* tuber α-amylase is summarized in Table 1. Chromatography of the crude enzyme on DEAE-cellulose column gave a single peak of activity before addition of NaCl (Fig. 1). The active peak (F-1) was pooled and concentrated that raised the purity of the enzyme to 19.6-fold. Purity of the pooled fraction (F-1) was checked by slab gel electrophoresis (Fig. 2) that gave multiple bands, indicating that F-1 contained more than 1 protein. Finally, CM-cellulose chromatography gave a single peak of activity at 0.15 M NaCl (Fig. 3). Active peak fractions were pooled and concentrated for characterization. This final step provided about 110-fold purification of the enzyme. The purified enzyme was homogeneous on SDS-slab gel electrophoresis, giving a single protein band (Fig. 4).

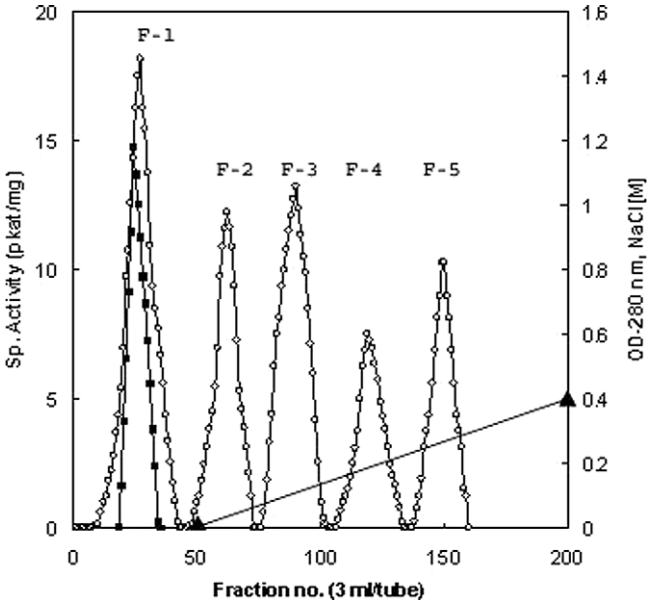

Fig. 1. Elution profile of DEAE-cellulose chromatography. Symbols: (○) OD at 280 nm; (■) enzyme activity; (▲) NaCl gradient.

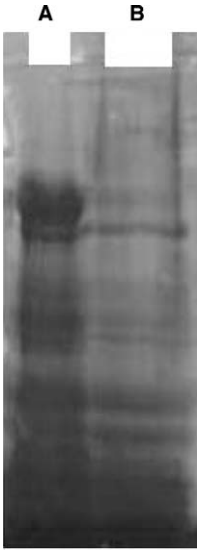

Fig. 2. Polyacrylamide slab gel electrophoresis of crude enzyme and F-1 fraction of DEAE-cellulose chromatography. Lanes A and B contained crude protein and F-1 fraction, respectively.

Table 1
Summary of purification of α-amylase from *P. erosus* L. tuber

Purification steps	Total protein (mg)	Total activity (μkat)	Specific activity (μkat/mg)	Yield (%)	Purification fold
Crude extract	430	825	1.9	100	1
DEAE-Cellulose chromatography	10.2	380	37.3	46.1	19.6
CM-Cellulose chromatography	0.9	188	209	22.8	110

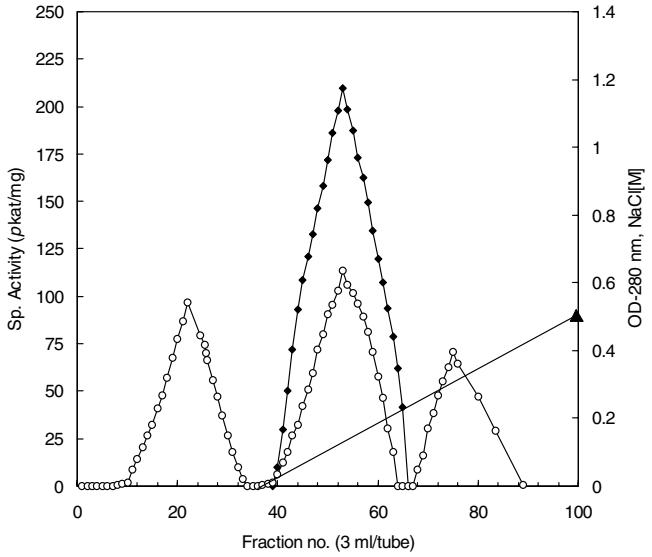

Fig. 3. Elution profile of CM-cellulose chromatography. Symbols: (○) OD at 280 nm; (■) enzyme activity; (▲) NaCl gradient.

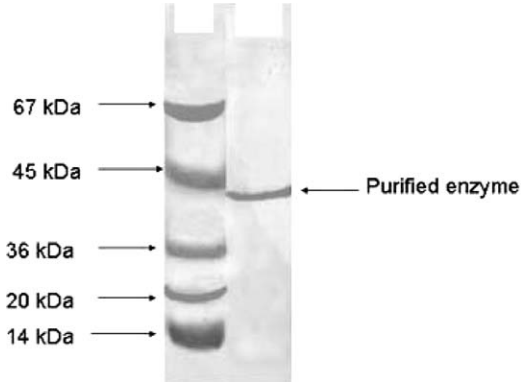

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified α-amylase and marker proteins for the determination of molecular weight of the enzyme.

Although the yield was low, the specific activity of the enzyme increased appreciably at each purification step. The decrease in yield may be due to denaturation of the enzyme during purification or to some other reasons. The specific activity of the purified *P. erosus* α-amylase compares well with other highly purified endoamylases (Feller et al., 1992).

3.2. Properties of the enzyme

SDS-PAGE of the purified α-amylase, with and without 2-mercaptoethanol treatment, revealed a single protein band of the same mobility, suggesting that the enzyme consisted of a single polypeptide chain. Reference to the relative mobility of the molecular weight marker proteins, run in parallel with this enzyme on SDS-PAGE (Fig. 4), gave a molecular weight of 40 kDa for the enzyme. The molecular weight of *P. erosus* tuber α-amylase is in the same range (38–45 kDa) as previously reported for α-amylases from other sources (Beers & Duke, 1990; Lin, Chyau, & Hsu, 1998).

P. erosus tuber α-amylase showed optimum activity at pH 7.3 (Fig. 5). The activity was found to decrease gradually at acidic pH, but it decreased rapidly at alkaline pH. Similar optimum pH (7.0) was observed for α-amylase from vine shoot inter-nodes (Berbezy, Legendre, & Maujean, 1996). Beers and Duke (1990) reported the optimum pH 5.5–6.5 for α-amylase from shoots and cotyledons of pea (*Pisum sativam* L.) seedlings, which is lower than that of the present finding. The enzyme was stable over a narrow range of pH, between 6.0 and 8.0, for 24 h incubation at 4 °C; the residual activity at pH 9.0 was 35% (Fig. 5).

The enzyme maintained above 50% activity over a temperature range of 20–60 °C with the optimum at 37 °C (Fig. 6). The activity increased sharply with gradual increase in temperature up to 37 °C while it gradually declined with further rise in temperature, indicating loss in the active conformation of the enzyme. The enzyme was only 20% active at 70 °C. These results are in accordance with those previously reported for amy-III, one of the two allozymes of α-amylase from *S. oryzae* (Abe, Chib, & Nakajima, 2002). The enzyme was stable at temperatures up to 40 °C for 30 min incubation (Fig. 6). Rapid inactivation occurred above 40 °C.

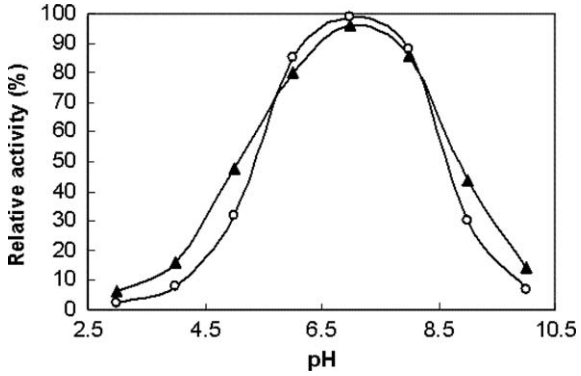

Fig. 5. Effects of pH on the activity and stability of *Pachyrhizus erosus* L. tuber amylase. (▲) optimum pH; and (○) pH stability.

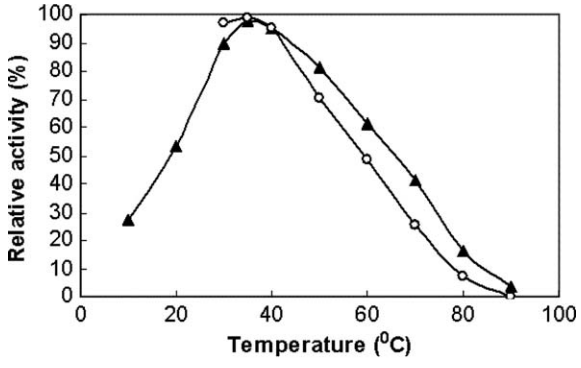

Fig. 6. Effects of temperature on the activity and stability of *Pachyrhizus erosus* L. tuber amylase. (▲) optimum temperature; and (○) temperature stability.

3.3. Kinetic constants

Kinetic studies were carried out under standard conditions. Apparent K_m and V_{max} values, determined from Lineweaver–Burk plots, were 0.29% and 0.37 μM/min/mg protein, respectively, for starch as substrate. This K_m value is similar to that reported for two allozymes from *S. oryzae* (Abe et al., 2002), but almost double of that reported for the amylase preparation from *R. dominica* (Baker, 1991).

3.4. Glycoprotein test and sugar content

The purified enzyme gave a yellow-orange colour in the presence of phenol-sulfuric acid, indicating that the enzyme contained sugar and hence was a glycoprotein. The sugar content of the enzyme was calculated to be 2.6%. It is reported that the extra cellular α-amylase from the yeast *S. alluvius* was a glycoprotein (Moranelli, Yaguchi, Calleja, & Nasim, 1987). The presence of carbohydrate in porcine pancreatic amylase was also observed (Beaupoil-Abadie, Raffalli, Cozzone, & Marchis-Mouren, 1973).

3.5. Substrate specificities

The data on substrate specificity of the enzyme are summarized in Table 2. In general, high-molecular-mass substrates containing the α-1,4-linkage were better substrates for the enzyme. The relative rate of hydrolysis of the polymeric substrate decreased with decreasing percentage of α-1,4-linkages and increasing percentage of α-1,6-linkages in the substrate, suggesting that the enzyme prefers high-molecular-mass, amylose type material as the substrate. It hydrolyzed amylose at rates similar to those obtained with soluble starch, but it was considerably less active on amylopectin and showed no effect on maltose and maltotetraose. Beers and Duke (1990) reported similar substrate specificity for pea α-amylase. Nakakuki, Azuma, and Kajnuma (1984) reported that *B. licheniformis* α-amylase was capable of cleaving oligosaccharides with a minimum degree of polymerization of 6 (maltohexose). Saito (1973) reported that *B. licheniformis* α-amylase could cleave oligosaccharides with a minimum degree of polymerization of 4 (maltotetrarose).

Table 2
Substrate specificity of *P. erosus* L. tuber α-amylase

Substrate	Glycosidic bond	Relative activity
Soluble starch	Glu α 1,4 Glu–Glu 1,6 Glu.	100
Amylose	Glu α 1,4 Glu	95
Amylopectin	Glu α 1,4 Glu–Glu 1,6 Glu	60
Maltotetraose	Glu α 1,4 Glu	nd
Maltose	Glu α 1,4 Glu	nd
Potato starch	–	92
Maiz starch	–	85
Wheat starch	–	82
Rice starch	–	78

The activity for soluble starch was taken as 100%. Abbreviation: nd, not detected.

3.6. Effects of EDTA and metal ions

Effects of mono and divalent metal ions on the purified α-amylase was determined at pH 7.3. The monovalent cations tested showed slight negative effects, except Na^+, while the divalent cations, except Ca^{2+} and Mn^{2+}, exhibited a strong inhibitory effect (Table 3). The addition of Zn^{2+}, a known inhibitor of plant α-amylase (Savchenko, Vielle, Kang, & Zeikus, 2002), to the purified enzyme resulted in a 90% loss of activity. Ranwala and Miller (2000) reported that Hg^{2+} and Ag^+ completely inhibited the enzyme at concentrations of 2.0 mM. Shaw and Ou-Lee (1984) reported α-amylase to be strongly inhibited by Cu^{2+} and moderately by Li^+.

Activity of the enzyme almost doubled in the presence of 100 mM Ca^{2+}, suggesting that calcium is needed for the optimum activity and stability of the enzyme. It is reported that calcium is required for α-amylase secretion and synthesis in *P. furiosus* (Savchenko et al., 2002). Ca^{2+} is required for α-amylase activity; the inhibitory effects of other divalent cations might be due to the competition for calcium binding sites while monovalent cations and Mg^{2+} might be poor competitors for calcium binding (Shaw & Ou-Lee, 1984). Mn^{2+} and Fe^{3+} also highly enhanced the activity of this enzyme.

Amylase activity was almost completely abolished by 100 mM EDTA, indicating the metal ion requirement of this enzyme (Table 3). Boyer and Ingle (1972) and Berbezy et al. (1996) reported similar inhibitory effects of EDTA on the activity of α-amylase. Further, the activity of the enzyme only slightly changed in the presence or absence of 10 mM cysteine, an SH-dependent β-amylase inhibitor. Strong inhibition by Zn^{2+} and EDTA and slight inhibition, by cysteine, of the enzyme clearly revealed that the purified *P. erosus* tuber amylase was of the α-type.

Table 3
Effects of EDTA and metal ions on *P. erosus* L. tuber α-amylase activity

Reagents	Relative activity (%)						
	1 mM	2 mM	5 mM	10 mM	20 mM	50 mM	100 mM
None	100	100	100	100	100	100	100
EDTA	43.8	35.1	28.7	21.4	15.2	3.9	1.1
Na^+	110	115	126	140	146	157	165
K^+	93.2	89.1	87.6	82.5	70.2	75.2	70.8
Ag^+	96.0	93.3	85.1	80.3	75.6	71.4	68.5
Ca^{2+}	119	128	142	158	168	178	195
Mg^{2+}	96.4	92.3	82.2	76.5	74.2	72.6	67.4
Cd^{2+}	88.0	78.0	61.0	48.6	43.7	40.8	38.7
Zn^{2+}	71.7	65.0	56.7	42.5	32.1	21.6	10.6
Cu^{2+}	77.4	69.6	48.0	35.6	27.9	20.4	8.5
Hg^{2+}	91.0	85.8	74.5	62.3	55.5	50.5	38.3
Li^{2+}	93.0	87.0	64.0	50.5	45.8	42.3	28.6
Mn^{2+}	124	134	140	141	144	152	151
Fe^{2+}	95.0	80.0	42.0	30.2	26.3	22.4	10.2
Fe^{3+}	113	120	126	156	161	168	171
Cysteine	92.6	88.5	85.4	84.2	–	–	–

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