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Characterization of β-amylase From Lentil (*Lens esculenta* L.) Cotyledons

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Abstract: In the cotyledons of germinating Lentil (*Lens esculenta* L.) at 15 days the abundant amylolytic activity was found to be β-amylase (E.C. 3.2.1.2, α-1-4-glucan maltohydrolase). β-amylase from germinating Lentil cotyledons was purified to homogeneity for study of enzyme characteristics. The purification steps included ammonium sulphate precipitation followed by DEAE-cellulose chromatography and hydroxylapatite chromatography. By successive steps of $(NH_4)_2 SO_4$ fractionation, DEAE-cellulose chromatography and HA filtration, the purity of the enzyme from lentil increased 108.5 fold with an overall yield of 32.2% with specific activity of 575 U mg^{-1}. The end product of α-1, 4-glucan degradation was maltose. The β-amylase was most active at pH 6.1 (soluble starch substrate) and 40°C. Km for Lentil cotyledons β-amylase for soluble potato starch was 1.67 milligrams per milliter (mg ml^{-1}). The molecular weight of the enzyme was estimated to be 58 kilodaltons (kDa) by both gel filtration and sodium dodecyl sulphate gel electrophoresis. Heavy-metal ions Cu^{2+}, Hg^{2+} and Ag^{+} at 1.0 mM concentration and p-hydroxymercuribenzoic acid and N-bromosuccinimide at 0.4 mM concentration inhibited the enzyme activity. Lentil β-amylase is competitively inhibited by its end product, maltose, with a K_i of 8.4 mM. Less branched or no-branched (amyloses) were better substrates for Lentil cotyledons β-amylase than moderately branched glucans (amylopectin) or highly branched (glycogens) glucans.

Key words: Lentil (*Lens esculenta* L.) cotyledon, enzyme characterization, starch degradation

INTRODUCTION

β-amylase an enzyme involved in starch degradation is present in some species of higher plants and microbes[1]. It has long been known that the mature ungerminated kernels of cereals (barley and rye) content activities of β-amylase. When the seed germinate, this activity increases considerably[2,3]. The molecular characterization of β-amylase has been mainly carried out on enzyme purified from the organ enriched in starch. β-amylase, considered as one of the enzymes, which degrade starch, may play a role in the mobilization of starch during germination or the sprouting of tubers[4-6]. Extensive studies have been done on the isolation of β-amylase enzyme from seeds[7-11] and tubers[12-17]. In contrast, relatively little information is available on the purification and the molecular characterization of β-amylase from the green leaves of higher plants[18-20]. In the mustard (*Sinapis alba*) seedlings, β-amylase is the sole amyolytic enzyme, as these seedlings characteristically lack α-amylase[21]. While β-amylase is activated from a pre-existing zymogen from during the germination of cereal seeds, in mustard seedlings it is synthesized *de novo* in the cotyledons under the influence of light[21, 22]. There are few overviews dealing extensively with cereal β-amylases[1,23] and a few research works is concerned exclusively with these enzymes[24]. The need for new plant sources of β-amylase still remains[16], for formulating of enzymatic complex's of starch degrading enzymes for biotechnological applications. The amylolytic activities in lentil seedlings have not been studied. This work is an attempt to fill that gap.

In this study we describe the molecular properties of homogeneously purified β-amylase from germinating seedling of lentil that is synthesized by *de novo* in the cotyledons under the influence of light and compared its enzymatic properties with those of other higher plant β-amylases.

MATERIALS AND METHODS

Chemicals: All chemicals were of analytical grade and purchased from Sigma Inc., USA. Soluble potato starch, amylopectin and amylose were also obtained from Sigma Co. p-hydroxymercuribenzoic acid and N-bromosuccinimide was purchased from local market.

Plant material: Lentil (*Lens esculenta* L.) plants were cultivated in a growth chamber under a 40 w fluorescence bulb. Day and night temperature was 25 and 20°C, respectively. Plant material (cotyledons with young

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leaves) were harvested after 7, 15, 20, 25 and 30 days and stored at 6-8°C in a refrigerator.

Enzyme extraction: Twenty grams of cotyledons with young leaves were cut into small pieces and homogenized with 200 ml of de-ionized water in a grinder machine (Jamboo, India) at 4°C for 15 min. The homogenate was filtered through one layer Miracloth and centrifuge at 10,000 g for 15 min. at 4°C to remove insoluble materials.

Purification of β-amylase: All enzyme purification steps were carried out at 4°C. The crude extract was initially fractionated by ammonium sulphate precipitation. Protein precipitated at 41-80% $(NH_4)_2SO_4$ saturation was used for further purification. After centrifugation at 10,000 g for 15 min the precipitated pellets was re-suspended in 50 ml of 20 mM acetate buffer, pH 5.4 and dialyzed against 20 mM acetate buffer, pH 5.4 for 24 h and concentrated to 30 ml by polyethylene glycol.

DEAE-cellulose column chromatography: The concentrated dialyzed enzyme solution was applied to a DEAE cellulose column (35x1.25 cm) previously equilibrated with 20 mM acetate buffer (pH 5.4) and eluted with the same buffer at the flow rate 25 ml h^{-1}, containing linear gradient of NaCl (100-500 mM). The Enzymatically active fractions (No. 21-28) collected and dialyzed against 10 mM phosphate buffer, pH 6.1 and concentrated to 10 ml by freeze dryer.

Hydroxylapatite chromatography: The concentrated dialyzed enzyme solution was applied to a hydroxylapatite column (20 x2.00 cm) glass column previously equilibrated with 10 mM phosphate buffer (pH 6.1) in a cold room at 4^0C. The column washed 2-3 times with the same buffer at the flow rate 25 ml h^{-1}. The enzymatically active fractions (No.31-37), which were adsorbed on hydroxylapatite column eluted with same buffer at the flow rate 25 ml h^{-1}, containing linear gradient of NaCl (100-500 mM). Active fractions from hydroxylapatite step were pooled, concentrated by freeze drier and applied for molecular weight determination by gel filtration and electrophoresis.

Determination of β-amylase activity: One milliliter of enzyme solution was added to 1ml of 1% soluble starch containing 0.1 M acetate buffer (pH 5.1) and the mixture was incubated at 40°C for 15 min. The amount of reducing sugars produced was determined calorimetrically by the Somogyi method[25]. One unit of enzyme activity was defined as the amount, which catalyzed the formation of 1 μmol of maltose per min. under the assay conditions.

Protein concentration was determined by Lowry's phenol method[26], using crystalline BSA as the standard.

Effect of pH and temperature on lentil β-amylase: Optimum pH and the pH-stability of the enzyme are shown in Fig. 4. The optimum pH was at 6.1. To examine the pH-stability of the enzyme, the enzyme was incubated at 40°C for 1h in phosphate buffers of various pHs and the residual activity was measured at pH 6.1. The enzyme is stable over the range of pH 5-7.

Optimum temperature and thermostabitity of the enzyme are shown in Fig. 5. The optimum temperature was at 40°C under the reaction conditions employed (reaction for 15 min). To measure the thermostabitity of the enzyme, the enzyme was incubated at pH 6.1 at various temperatures for 15 min. and the remaining activity was determined by the standard method. Over 90% of the enzyme activity remained at temperature below 45°C but the enzyme was inactivated at 50°C.

Influence of metal ions and chemical reagents: The effects of metal ions and inhibitors on the enzyme activity were examined. Enzyme was added to the substrate solution preincubated with a reagent at 40°C for 5 min and residual activity (%) was determined. As shown in Table 3, $FeCl_2$, $HgCl_2$, $CuCl_2$ and $SnCl_2$ reduced the enzyme activity. The enzyme was also inhibited by SH-inhibitor such as p-chloromercuribenzoic acid (PCMB) and adding reducing agents such as cysteine or mercaptoethanol abolished the inhibition.

Determination of molecular weight: Gel Filtration Column: The column was prepared with Sephadex G-200 (Pharmacia) in a 35 x 1.25 cm glass column. Procedures for hydrating the gel and packing the column and determination of void volume were done as described by Cooper[27] and Rendina[28]. The bed dimension was 25x1.0 cm and it was fitted with flow adapters.

First, the elution volumes of protein standards of known molecular weights, namely, lysozyme (14 kDa); soybean trypsin inhibitor (21.5 kDa); Albumin (bovine, 67 kDa); phosphorylase b (92.5 kDa) and β-galactosidase (116 kDa) were determined on the Sephadex G-200 column from their elution profiles according to the method described by Cooper[27]. A selectively curve was then prepared by plotting their partition coefficients, K_{av}, against the logarithm of their molecular weights. The partition coefficient of the purified β-amylase was calculated from the previously determined elution volume and interpolated on the curve to obtain its molecular weight.

Sodium Dodecyl Sulfate (SDS): Polyacrylamide Gel Electrophoresis was performed according to the method of Laemmli[29]. Prepared enzyme solution and the following solution containing standard proteins were applied to the PAGE. The standard proteins (low) of BioRad Laboratories were lysozyme (14 kDa), trypsinogen (24 kDa), albumin (chicken, 45 kDa), albumin (bovine, 67 kDa), phosphorylase b (92.5 kDa) and β-galactosidase (116 kDa) PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A. The protein band on the gel was dyed by use of 0.25% Coomassie brilliant blue R-25 (CBB) solution containing 50% methanol and 10% acetic acid.

Determination of Km value: The initial velocity is equal to the amount of product formed per unit time. The initial velocity (V_i) was determined by measuring quantitatively the amount of one of the product at various times[30].

RESULTS AND DISCUSSION

From time course study it was found that β-amylase from germinating lentil seeds (seedlings with fresh leaves) showed their maximum activity after 15 days seedlings and then declined rapidly (Fig. 1). In higher plants, the molecular characterization of β-amylase has been mainly carried out on the enzyme isolated from the storage organs. Table 1 represents the successive steps of $(NH_4)_2 SO_4$ fractionation of Lentil cotyledons β-amylase. By successive steps of $(NH_4)_2 SO_4$ fractionation, DEAE-cellulose and hydroxylapatite column chromatography, the purity of the enzyme from lentil increased 108.5 fold with an overall yield of 32.2% with specific activity of 575 U mg^{-1} (Table 2).

Table 1: $(NH_4)_2SO_4$ Fractionation of lentil β-amylase

$(NH_4)_2SO_4$ (%)	Activity precipitated (units)	Protein (mg)	Specific activity (units mg^{-1})
0-25	30	9.50	3.1
26-40	60	11.45	5.2
41-60*	365	21.20	17.2
61-70 *	425	28.90	14.7
71-80*	460	29.90	15.4
81-90	69	21.00	3.2

* Fraction pooled

Table 2: Purification of lentil cotyledons β-amylase. The values are the average from three purifications

Fraction	Total protein (mg)	Total activity (Unit)	Specific activity (U mg^{-1})	Yield (%)	Purification (Fold)
Crude extract	265.0	1425	5.3	100	1
$(NH_4)_2SO_4$	80.0	1250	15.6	87.7	2.94
DEAE-cellulose	15.8	890	56.3	62.4	10.6
Hydroxylapatite Column	0.8	460	575.0	32.2	108.5

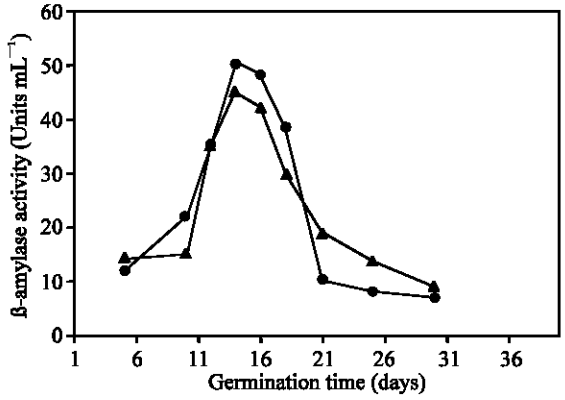

Fig. 1: Time course study of crude β-amylase extract (-▲-) and Partial purified (-●-) β-amylase from lentil cotyledon.

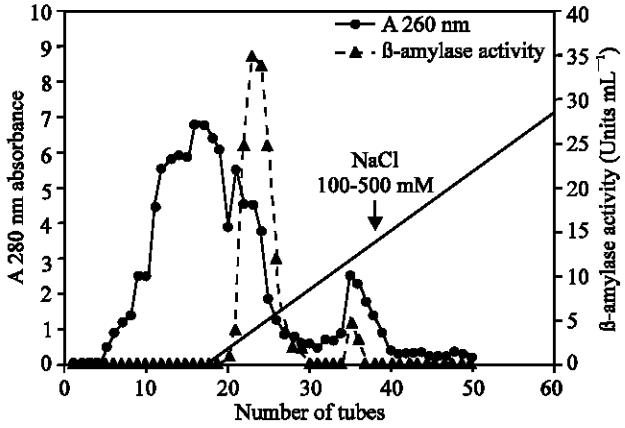

Fig. 2: Elution profile of lentil cotyledons proteins with amylolytic activities from DEAE cellulose column (35x1.25 cm) previously equilibrated with 20 mM acetate buffer (pH 5.4) and eluted with the same buffer at the flow rate 25 ml h^{-1}, containing linear gradient of NaCl (100-500 mM.)

Thirty milliliters of dialyzed and concentrated solution that had originated from 20g of lentil cotyledons with young leaves were charged on a DEAE-cellulose column (35x1.25 cm) equilibrated with 20 mM acetate buffer (pH 5.4). The column was washed with the same buffer and the adsorbed enzyme was eluted from the column with a linear gradient of sodium chloride 100-500 mM in the same buffer (Fig. 2). The Enzymatically active fractions (No. 21-28) were collected, dialyzed against 10 mM phosphate buffer, pH 6.1 at 4°C for 48h, concentrated (10 ml) with polyethylene glycol and re-chromatographic on a Hydroxylapatite column.

The enzyme solution was applied to a Hydroxylapatite column (20x2.0 cm), previously equilibrated with 10 mM phosphate buffer (pH 6.1).

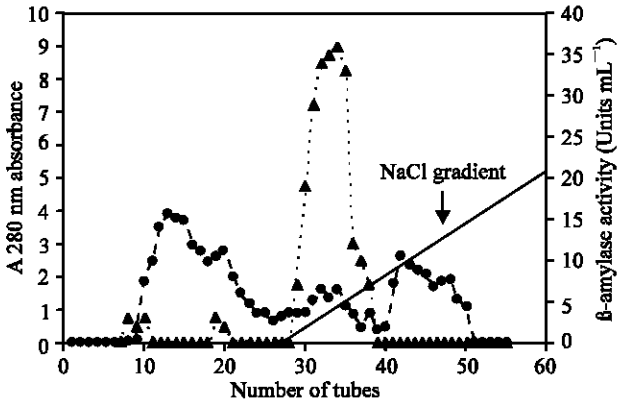

Fig. 3: Elution pattern of lentil cotyledons α-amylase from DEAE-cellulose column on Hydroxylapatite column (20 x 2.00 cm) glass column previously equilibrated with 10 mM phosphate buffer (pH 6.1), containing NaCl gradient. Flow rate 25 ml h^{-1}. β-amylase activity (-▲-) and (-●-) Protein concentration

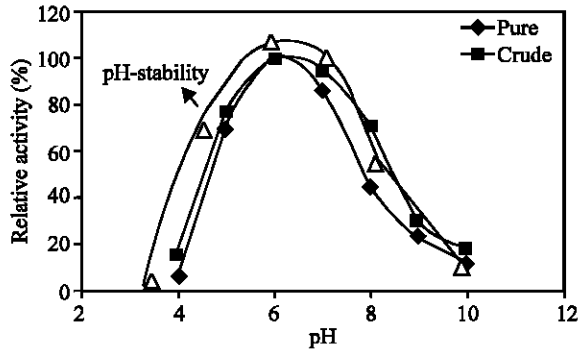

Fig. 4: Optimum pH and pH-stability of lentil cotyledons β-amylase

Optimum pH: The reaction mixtures consisting of 500 µml of the enzyme solution, 1 ml of 1% soluble starch and 500 µml of 0.1 M acetate buffers (pH 5.1) at various pH, were incubated at 40°C for 15 min.

pH-stability: The enzyme solution (250 µml) was mixed with 250 µml of 10 mM acetate buffer of various pHs and incubated at 40°C for 50 min. Then 500 µml of 10 mM acetate buffer and 1 ml of 1% soluble starch were added and the activity was measured in the usual way.

Elution was carried out with the same buffer containing 100-500 mM sodium chloride gradient at the flow rate 25 ml h^{-1}. Three major peaks were discernible from the Hydroxylapatite column, eluting profile shown in (Fig. 3.), but maximum activity of the enzyme was restricted in the small peak (fraction No 31-37). On column chromatography only one active peak was found and it

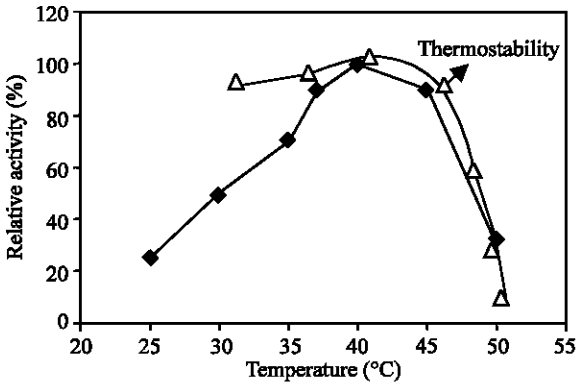

Fig. 5: Optimum temperature and Thermostability of lentil cotyledons β-amylase

Optimum temperature: The reaction mixtures consisting of 1ml of the enzyme solution, 1 ml of 1% soluble starch containing 0.1 M acetate buffer (pH 5.1) and the mixture was incubated at 40°C for 15 min

Thermostability: The activity was measured in the way at 40°C after incubation of the enzyme solution at various temperatures for 15 min.

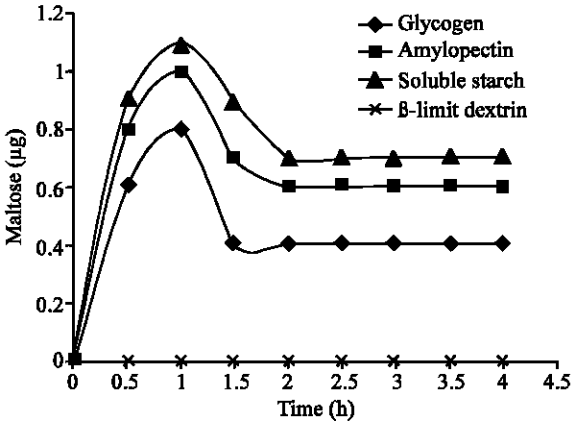

Fig. 6: Substrate specificity of β-amylase from lentil

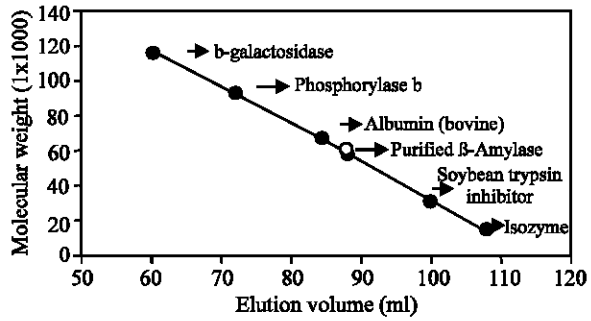

Fig. 7: Measurement of Molecular Mass of the Purified Amylase by gel filtration (Sephadex G 200)

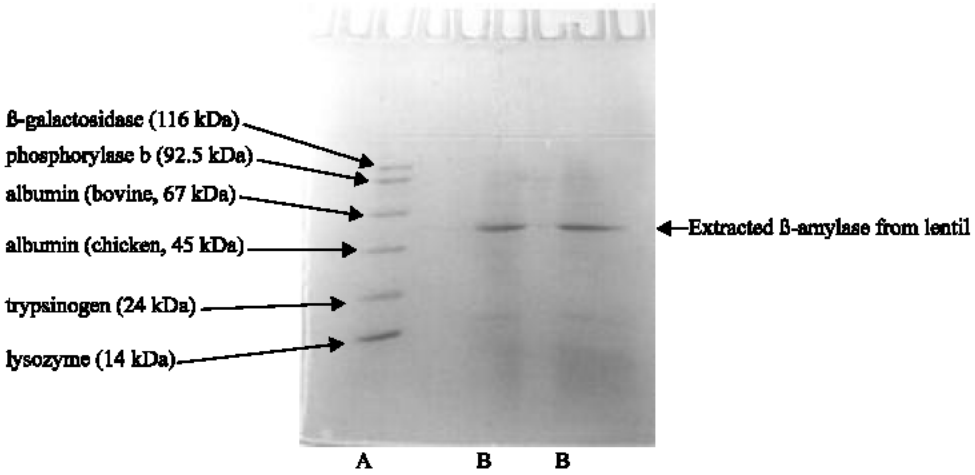

Fig. 8: Photographic representation of the sodium dodecyl sulfate polyacrylamide slab gel electrophoresis.
A = Marker Protein B = β-Amylase after DEAE cellulose and hydroxylapatite column

Table 3: Effect of metal ions and chemical reagents on lentil cotyledons β-amylase

Metal and Chemical reagent	Concentration (mM)	Residual activity (%)
None	-	100
$CaCl_2$	10	96
$MgCl_2$	10	80
$ZnCl_2$	10/1	94
$FeCl_3$	1/10	102
$FeCl_2$	1	31
$HgCl_2$,	1	14
$CuCl_2$	1	18
$SnCl_2$	1	32
p-Chloromercuribenzoic acid (PCMB)	1/10	0

Table 4: Lentil cotyledons β-amylase substrate specificity
all substrates were at an initial concentration of 2% (w/v)

Substrate	Activity [μmol min^{-1} mg^{-1} protein] (% Starch control)
Control (Soluble potato starch)	286 (100)
Amylopectin (potato)	143 (50.0)
Amylose (potato)	304 (106)
Amylose (maize)	292 (102)
Glycogen (mussel)	124 (43.2)
Maltose	0 (0)
β-Dextrin (potato starch)	0 (0)

seemed that no isozymes of this enzyme exist in lentil column chromatography has been used successfully by many workers for the purification of proteins[31, 32].

Several features of purified lentil cotyledons β-amylase distinguished it form β-amylase from storage organs. The pH optima (6.1- 6.2) of lentil cotyledons β-amylase (Fig. 4) is more towards neutral pH than sweet potato or barley seeds which ranges between pH 4 and 5[4, 33, 34].

Optimum temperature and thermostability of the enzyme are shown in Fig. 5. The optimum temperature was at 40°C under the reaction conditions employed (reaction for 15 min).

The purified lentil β-amylase also shows many properties in common with other β-amylases. The substrate specificity of lentil β-amylase is akin to that of other β-amylases[1,4]. Lentil β-amylase hydrolysed amylose, amylopectin and glycogen, but did not digest β-limit dextrin. The amylose was digested at a faster rate than amylopectin and glycogen (Table 4 and Fig. 6).

The Lentil cotyledons β-amylase was inactivated by heavy metal ions and by SH-inhibitor such as p-Chloromercuribenzoate (PCMB), the activity being restored by thiol compounds such as cysteine (Table 3). This result indicates that an SH group exists in the molecular structure, as in other plant β-amylases. Plant β-amylase has been reported to require free sulfhydryl groups for activity and is inhibited by heavy metals as well as other thiol binding reagents[1,35]. Lentil cotyledons β-amylase on gel filtration as well as on SDS-PAGE showed a molecular weight of 58 kDa. Hence Lentil cotyledons β-amylase is a monomer (Fig. 7 and 8). This value was in accordance with molecular weights of B-amylase isolated from pea epicotyl[36], barley[37] and soybean[38].

As determined by the Lineweaver-Burk method, the apparent Michaelis constant (K_m) of Lentil cotyledons β-amylase for soluble potato starch was 1.67 mg ml^{-1}. Starch hydrolysis by Lentil cotyledons β-amylase was competitively inhibitated by the end product, maltose, with a K_i of 8.4 mM at pH 6.1. Nomura *et al.*[39] reported competitive inhibition by maltose of soybean β-amylase with K_i values of 5.8 mM at pH 5.4 and 4.1 mM at pH 8.0. Sweet potato β-amylase was also competitively inhibited by maltose with a K_i of 6.0 mM[40]. The apparent Km values of β-amylase so far described were in the range of 0.88- 2.63[16].

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