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REGULAR ARTICLE

# PARTIAL CHARACTERIZATION OF α-AMYLASE FROM GERMINATING LITTLE MILLETS (*PANICUM SUMATRENSE*)

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## SUMMARY

α-Amylase, a starch splitting enzyme, was purified to homogeneity from little millet (*Panicum sumatrense* L. Roth ex Roem. et Schult.) cotyledons excised from 3-day-old seedlings by successive chromatography on DEAE-cellulose and Sephadex G-150. Purification achieved was 10.15 fold from the crude extract with a yield of 29% giving a final specific activity of 1001U/mg protein. SDS-PAGE showed a molecular weight of 46 kDa for the enzyme. The enzyme was characterized in terms of pH optimum and stability, temperature optimum and stability, activation energy, $K_m$ and $V_{max}$. The enzyme displayed optimum activity at pH 5.0 and 50°C with an apparent $K_m$ value of 1.6 mg for soluble starch as substrate and $V_{max}$ 1388 units/ min/mg protein. The energy of activation (Ea) for the enzyme-catalyzed reaction was 9.7 kcal./mole. Significant enhancement in the enzyme activity was observed in the presence of metal ions like $Ca^{2+}$ and $Ba^{2+}$ while metal ions such as $Fe^{2+}$, $Hg^{2+}$ and $Al^{3+}$ completely inactivated the enzyme. Incubation of the enzyme with 10mM EDTA for 30 min at 45°C results in complete loss of activity.

**Key words:** α-Amylase, Little millet, Characterization, *Panicum sumatrense*

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## 1. Introduction

α-Amylase, an endoenzyme (1,4-D-glucan glucanohydrolase, E.C.3.2.1.1) is a key enzyme hydrolyzing reserve starch in the endosperm of germinating cereals (Machaiah and Vakil, 1984). α- Amylases catalyze the hydrolysis of internal α-1, 4-O-glycosidic bonds in polysaccharides with retention of α-anomeric configuration in the products. α-amylases are ubiquitous in nature and have been isolated, purified and characterized from a number of animal, plant, fungal as well as bacterial sources (Kumar *et al*., 2009). Starch depolymerization by amylases is the basis for several industrial processes such as preparation of glucose syrups, brewing and bread making. Cereal α-amylases play a very important role in the starch metabolism in developing as well as germinating cereals and have gained importance in supplementary foods, breweries and starch saccharification (Muralikrishna and Nirmala, 2005; Adewale *et al*., 2006). The purpose of this study is to isolate α-amylase from germinating little millet and compare its properties with other plant amylases.

Little millets are hardy and can grow in adverse conditions over various parts of India. Its resistance to drought and water logging assures farmers an adequate yield under conditions considered unfavourable for other cereals and makes little millet an important component in the diet. Little millets are rich in B-vitamins and have high nutritional value especially the presence of phosphorus and iron. As little millets contain no gluten, they are appropriate food for those with celiac disease or other forms of allergies/intolerance of wheat.

## 2. Materials and Methods

### Plant material

Little millet seeds (*Panicum sumatrense* L. Roth ex Roem. et Schult.), commercial variety,

were purchased from a local market in Paderu, Visakhapatnam, India. Grains were surface sterilized with 0.5% sodium hypochlorite solution washed several times and soaked in distilled water for 24h. Germination was carried out for 3 days at 25°C in dark on petri-dishes layered with moistened filter paper. Cotyledons were homogenized and used in the purification procedure. Deionized water was used throughout the experimentation.

**Determination of α-amylase activity and protein concentration**

α-Amylase was assayed according to the procedure of Bernfeld, *1955*. Gelatinized soluble starch (1%) in 0.05 M sodium acetate buffer (pH 5.0) was incubated with appropriately diluted enzyme at 45°C for 20 min. The reaction was stopped by the addition of (1ml) DNS reagent. One unit of enzyme activity was defined as µmol maltose equivalent released /min under the assay conditions. The specific activity was expressed as activity units /mg protein. Protein content was determined according to the method of Lowry et al.,*1951* using BSA as standard.

**Extraction and Purification of α-Amylase**

Cotyledons from 3-day old seedlings from germinating little millet were homogenized using mortar and pestle in 0.05 M sodium phosphate buffer (pH 6.9). Four milliliters of buffer was added per gram of fresh weight. α-Amylase was extracted for 1 h at 4°C. Extract was filtered through two layers of cheese cloth and centrifuged for 20 min at 4°C at 6,500×g. The crude extract was differentially precipitated with ammonium sulphate at its 0-30%, 30-60-% and 60-100% saturation. The fraction having maximum enzyme activity was dialyzed and further purified by loading onto DEAE-cellulose column. α-Amylase was eluted with increasing gradient of 0-0.3M NaCl in the eluting buffer. 3 ml fractions each were collected and monitored for protein as well as amylase activity. The fractions with high specific activity were pooled and subjected to gel permeation chromatography on Sephadex G-150 column and fractions (3ml) each were collected, monitored for protein (280nm) and amylase activity. Homogeneity and molecular weight of purified amylase was detected by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method according to Laemmli**,** ***1970***. Gels were stained with Coomassie brilliant blue R250. In order to determine molecular weight, SDS-PAGE molecular weight standards in the molecular mass range from 14.3 to 97.4 kDa were used.

**Determination of Kinetic Constants**

The activity of the enzyme was assayed in 0.05M sodium acetate buffer (pH 5.0) with varying substrate concentrations ranging from 0.2 to 2.0 mg/ml. $K_m$ and $V_{max}$ were calculated from LB plot (Lineweaver and Burk**,** 1934).

**pH optima and stability of purified α-amylase from little millet**

α-Amylase activities were determined at various pH values using different buffers such as glycine-HCl (2.0-3.0), sodium acetate (4.0-5.0), sodium phosphate (6.0-7.0), Tris-HCl (8.0-9.0) at 0.05M concentration with 1% soluble starch as substrate. The relative activity at different pH values was calculated, taking the maximum activity obtained as 100%.

Stability of α-amylase was determined by incubating purified amylase in different buffers at 4°C for 24h and the relative activities were measured under standard assay conditions.

**Temperature optima and stability of purified α-amylase from little millet**

α-Amylase activities were determined at a temperature range of 20-70°C (with an interval of 10°C) with 1% soluble starch as substrate. The relative activity at different temperatures was calculated, taking the maximum activity obtained as 100%.

The effect of temperature on enzyme activity was measured by incubating the purified enzyme at temperature range of 20-70°C for 20min. After heat treatment the enzyme solution was cooled and the relative activities were measured under standard assay conditions.

**Activation energy**

To determine the activation energy of the reaction catalyzed by the α-amylase an Arrhenius plot was made covering the temperature range 25-80°C.

**Influence of metal ions and inhibitors on amylase activity**

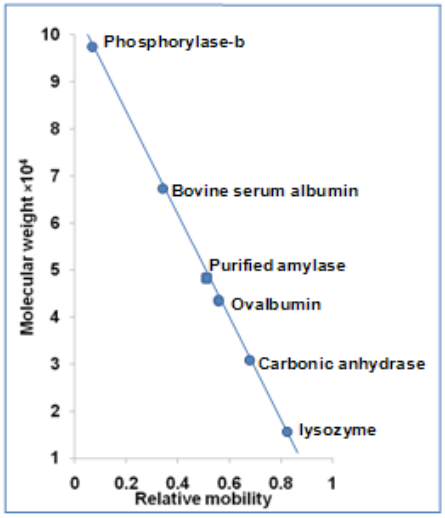
The influence of various metal ions and inhibitors on α-amylase activity was studied. Chlorides of various metals ($Ca^{2+}$, $Ba^{2+}$, $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Fe^{2+}$, $Al^{3+}$) at a final concentration of 5mM and 10 mM oxalic acid, citric acid and EDTA; 50mM iodoacetate, PCMB and carbodimide were used. The samples were preincubated for 30 min in 50mM acetate buffer (pH 5.0) at 45°C, supplemented with metal ion/inhibitor, and then enzyme assay was performed with soluble starch as substrate.

## 3. Results

**Extraction and Purification of little millet α-amylase**

The enzyme isolated from 3-day old germinating little millets was purified 10.15 fold. As a result of this procedure, a protein with specific activity of 1001 Units /mg was obtained whereas the recovery was 29%. The molecular weight as determined by SDS polyacrylamide gel method was 46 kDa. (Fig.1).

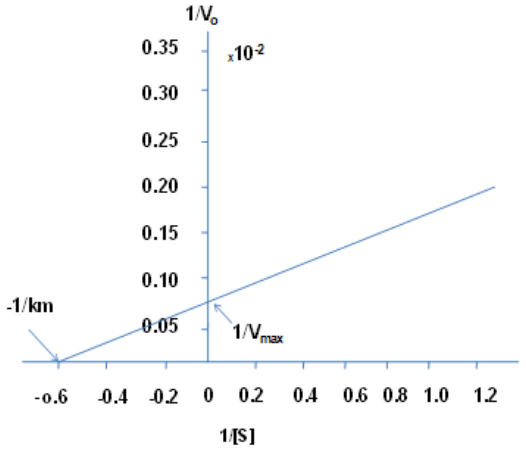
Fig.1 Molecular weight determination of little millet α-amylase by SDS-PAGE .Plot of the distance migrated against molecular weight of standard proteins




**Determination of kinetic constants**

Effect of different substrate concentrations on the initial velocity was calculated and the $K_m$ and $V_{max}$ for little millet α-amylase determined were found to be 1.6 mg/ml and $V_{max}$ 1388 units/min/ mg of protein, respectively (Fig.2).

Fig.2 Lineweaver-Burk plot of little millet α-amylase reaction velocities versus starch as substrate concentration. One milliliter of the reaction mixture containing 0.05M sodium acetate buffer pH 5.0, suitable aliquot of enzyme and concentrations of starch ranging from 0.2 to 2.0 mg/ml. Enzyme activity was assayed as per materials and methods




**pH optimum and stability**

The pH optimum for α-amylase activity was determined using buffers ranging in their pH values from 2.0-9.0. Soluble starch was used as substrate to determine the activity of amylase. From the results obtained it was revealed that the enzyme exhibited high activity at pH 5.0. Increasing or decreasing the pH resulted in decrease in the activity of the enzyme in the pH range studied (Fig. 3a).

The stability of α-Amylase was tested after preincubation at different pH values. The enzyme was found to be stable in the pH range of 4.0-7.5. However, the activity of the amylase decreased drastically below pH 3.0 after preincubation for 15 min (Fig.3b).

Fig.3a. pH optimum of little millet α-amylase. One milliliter of the reaction mixture containing 1% starch and suitable aliquot of enzyme and buffers at varying pH 0.05M glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0), sodium phosphate (pH 6.0-7.0), and Tris-HCl (8.0-9.0). Enzyme activity was assayed as per materials and methods

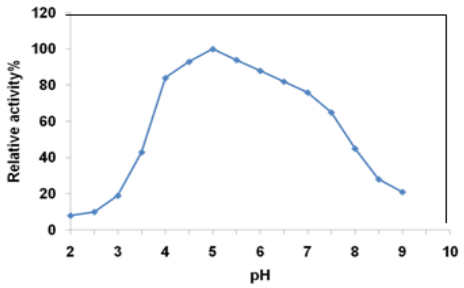
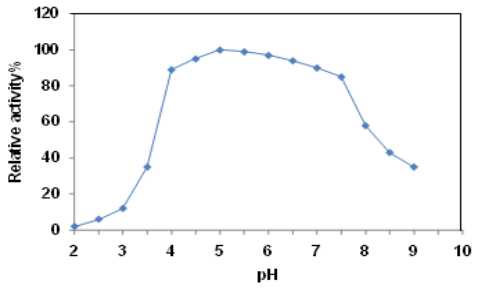



Fig.3b. Effect of pH on the activity of little millet α-amylase. Suitable aliquot of enzyme was preincubated in buffers at varying pH 0.05M glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0), sodium phosphate (pH 6.0-7.0), and Tris-HCl (8.0-9.0) for 24h. Relative activities were measured under standard assay conditions




## Temperature optimum and stability

α-Amylase exhibited maximum activity after 20 min incubation at 50°C. An increase in temperature of 10°C compared to the optimum resulted in a 21% decrease of activity in the presence of substrate soluble starch but further increasing of the temperature to 70°C resulted in further loss of enzyme activity (Fig.4a).

Fig.4 a Temperature optimum of little millet α-amylase. One milliliter of the reaction mixture containing 1% starch, 0.05M sodium acetate buffer pH 5.0, and suitable aliquot of enzyme. The enzyme activity was measured at varying temperatures ranging from 20-70°C

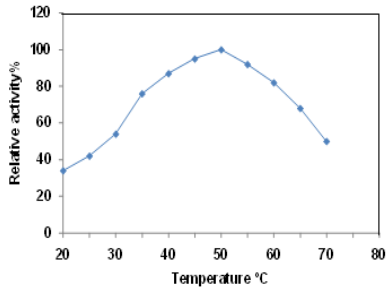
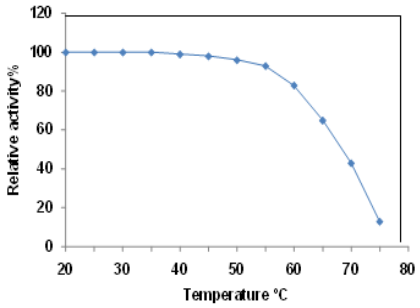



Fig.4.b. Effect of temperature on the activity of little millet α-amylase. Suitable aliquot of enzyme in 0.05M sodium acetate buffer pH 5.0 was preincubated at varying temperatures ranging from 20-70°C, followed by cooling. Relative activities were measured under standard assay conditions

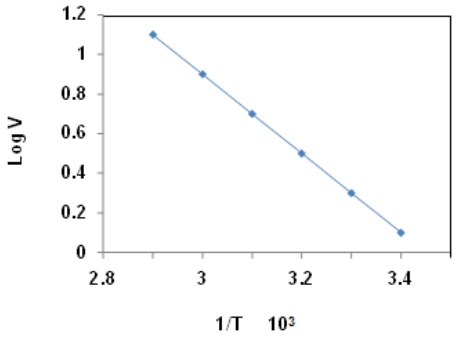



The thermal stability of amylase was carried out by preincubating at different temperatures and measuring the relative activities after 20 min. Eighty percent of hydrolysis activity was found to be in the temperature range of 20-60°C. Higher temperature (65°C and 70°C) resulted in considerably reduced stability of the enzyme. After 20 min preincubation at these temperatures, a 35% and 57% decrease in the activity was observed respectively (Fig.4b).

## Activation energy

The value of Ea obtained from the slope of the line is 9.7 kcal/mole (Fig.5).

Fig.5 Arrhenius plot of little millet α-amylase. Ea of the reaction catalyzed by the enzyme was assayed covering the temperature range 25-80°




## Influence of metal ions and inhibitors on the activity of amylase

Studies on the effect of ions of various metals on amylase activity revealed that the presence of calcium and barium ions enhance the enzyme activity by 15.0% and 9.0% respectively. No remarkable change in

enzyme activity was observed in the case of samples preincubated with magnesium, cobalt or manganese. Zinc and copper ions inhibited the enzyme activity by 80.0% and 92.5%, respectively, whereas preincubation of amylase with mercury, iron and aluminium ions resulted in a complete loss in the enzyme activity.

The activity of the enzyme was inhibited by 96% when treated with 10mM EDTA, 91% with 50mM carbodimide, 88% with 10mM citric acid and 85% with 10mM oxalic acid. Thiol inhibitors such as PCMB and iodoacetate had negligible effect on the activity, indicating that sulfhydryl group is not involved in the active site of the enzyme (Table-I).

Table I: Influence of metal ions and inhibitors on α-amylase activity. Suitable aliquot of enzyme was preincubated in 0.05M acetate buffer (pH 5.0) for 30 min at 45°C, supplemented with metal ion/inhibitor in the final concentration indicated. Relative activities were measured under standard assay conditions

| Metal ion/inhibitor | Metal ion/inhibitor Conc.(mM) | Relative Activity (%) |
|---|---|---|
| Control | 5.0 | 100 |
| $CaCl_2$ | 5.0 | 115.0 |
| $BaCl_2$ | 5.0 | 109.0 |
| $MgCl_2$ | 5.0 | 98.5 |
| $CoCl_2$ | 5.0 | 95.6 |
| $MnCl_2$ | 5.0 | 96.4 |
| $ZnCl_2$ | 5.0 | 20.0 |
| $CuCl_2$ | 5.0 | 7.5 |
| $FeCl_2$ | 5.0 | 0.0 |
| $AlCl_3$ | 5.0 | 0.0 |
| $HgCl_2$ | 5.0 | 0.0 |
| EDTA | 10.0 | 4.0 |
| Carbodimide | 50.0 | 9.0 |
| Citric acid | 10.0 | 12.0 |
| Oxalic acid | 10.0 | 15.0 |
| Iodoacetate | 50.0 | 92.4 |
| PCMB | 50.0 | 91.6 |

## 4. Discussion

It is well known that germination of cereals causes a marked increase in their amylase activities (Andrew and Bailey,1934) The activity of amylase in little millets increased from day 0 to day 3 where it exhibited its maximum activity. Denovo synthesis of α-amylase has been reported in germinating rice seeds, wherein α-amylase levels increased several hundred fold over a 4 day period (Okamoto and Akazawa, 1979; Mitsui *et al*., 1996). α-Amylase from malted sorghum has been reported to be purified 24.7-fold, with a recovery of 17.1%, using 0-

40% ammonium sulfate precipitation, ion exchange chromatography on DEAE cellulose and GPC on G-75 (Kumar *et al.*, 2005). In this study little millet amylase was purified to homogeneity, with a purification fold 10.15 and recovery of 29%, with a molecular mass of 46 kDa as determined by the SDS-PAGE. In general, the molecular weight of malt cereal enzymes ranged from 42-46 kDa (Greenwood and Milne, 1968). However, there are exceptions wherein a molecular weight 52 kDa was reported for amylases from malted barley (MacGregor, 1978) and 52-54 kDa for immature wheat (Marchylo *et al.*,1976).

The $K_m$ and $V_{max}$ for little millet amylase was determined for soluble starch to be 1.6 mg/ml and 1388 units/min/mg protein respectively. The $K_m$ value reported for the α-amylases from several other sources are very similar (Beers and Duke, 1990; Tripathi *et al.*,2007) and 0.57 mg and 1.3 mg starch/ml were reported from wheat (Fahmy et al.,2000).

The little millet amylase has an acidic pH optimum of 5.0 as do most plant amylases. Similar acidic pH range 4.5 to 6.5 for activity of amylase has been reported for amylases from finger millet (Nirmala and Muralikrishna, 2003b), shoots and cotyledons of pea (*Pisum sativum* L.) seedlings (Beers and Duke, 1990) and wheat (Kruger and Tkachuk, 1974), while slightly alkaline pH optimum was reported for amylase from *Pachyrhizus erosus* tuber (pH 7.3) (Noman *et al.*,2006). The enzyme was found to be stable in the pH range 4.0-7.5 and retained about 85% activity after 24 h of incubation at pH 7.5. However, the enzyme is inactive below a pH value of 3.0, indicating acid labile nature, a property characteristic of several cereal α-amylases (Thoma *et al.*, 1971). The pH stability of ragi amylases was reported to be in the range 5.0 to 9.0 and inactivated below pH 4.0 (Nirmala and Muralikrishna, 2003a).

The enzyme activity was optimal at 50°C. This value is comparable to the one reported for ragi α-2 amylase (Nirmala and Muralikrishna, 2003b). However, slightly higher temperature optima 55°C was reported for amylases from malted barley (MacGregor, 1978) and pearl millet (Adelaide and Marston, 1981). A temperature optimum of 60°C was reported for sorghum α-amylase (Kumar *et al.* 2005). Enzymatic activity measurements carried out at various temperatures indicated that purified little millet amylase is stable upto 60°C and started denaturing at 65°C after 20 min preincubation and lost about 35% of its activity at this temperature. This is in good agreement with the findings on amylase from wheat (Kruger and Tkachuk, 1974). Many factors such as the purity of the preparation, the presence of calcium as well as substrate and other stabilizers can affect the thermal stability of the amylase. The stabilizing effect of substrate can be attributed to the presence of small amounts of $Ca^{2+}$ present as impurities in the starch and this can stabilize conformation of the enzyme in a more rigid and stable form against denaturing conditions (Vihinen and Mantsilla, 1989)

The apparent energy of activation of little millet amylase was calculated to be 9.7 kcal./mole between 25°C to 80°C, which is in consonance with those reported from malted barley (MacGregor, 1978) and immature wheat (Marchylo *et al.*,1976) and a value of 10.8 kcal./mole was reported from *Vigna mungo* seeds (Koshiba and Minamikawa, 1981). Greenwood and Milne reported values of 11 and 14 for Ea at 25°C and 9°C for cereal amylases (Greenwood and Milne, 1968).

Cereal amylases are known to be metallo enzymes containing at least one $Ca^{2+}$ per molecule (Janeck and Belaz, 1992) and its number may go upto ten (Vihinen and Mantsilla, 1989). Enhancement of amylase activity by $Ca^{2+}$ ions is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which resulted in stabilization as well as maintenance of enzyme conformation. In addition, calcium is known to have a role in substrate binding (Sprinz, 1999). The inactivation by metal ions (Hg, Fe, Al, Cu, and Zn) may be due to their binding to either catalytic residues or by replacing the $Ca^{2+}$ from the substrate binding site of the enzyme (Nirmala and Muralikrishna, 2003b).

Since the general property of α-amylases is their content of calcium ions as a consequence, calcium-ion binding agents are good inactivators of enzyme of this type. The α-amylase purified from little millet proves no exception, since it is readily inactivated by EDTA. α-Amylase from cotyledons of germinating *Vigna mungo* seeds showed 50-90% loss of activity with 5mM EDTA (Koshiba and Minamikawa, 1981) and no activity was reported when pea cotyledon amylase was incubated with 5 mM EDTA (Swain and Dekker, 1966).

The inhibition by carbodimide suggests the possible presence of acidic amino acids such as aspartic and glutamic acids at the active sites (Nirmala and Muralikrishna, 2003b). Carboxylic acids such as oxalic and citric acids inhibit amylase by selective removal of $Ca^{2+}$ present in the enzyme (Nirmala and Muralikrishna, 2003a).

## 5. Conclusion

Amylases are important in many industrial processes. Keeping this in view, the search for α-amylase production from new sources is a continuous process. This study has demonstrated that the α-amylase purified from germinating little millets was found to be suitable for industrial use with respect to pH and temperature optima and stability, effect of activators, inhibitors, metal ions, and the apparent *K*m.

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