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PURIFICATION AND CHARACTERIZATION OF ALPHA AMYLASE FROM SEEDS OF PEARL MILLET (*Pennisetum typhoides*)

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Abstract: Alpha-amylase from pearl millet (*Pennisetum typhoides*) was purified to homogeneity through several steps involving change of pH (pH 7.0 to 6.0), lyophilization (-55 ^{o}C), polyethylene glycol precipitation (3-15%), Sepharose 6 B column chromatography and CM-cellulose column chromatography. The 24 fold purified enzyme had specific activity of 580 units/mg protein. Native PAGE revealed a single protein band and activity staining revealed that the band corresponds to alpha amylase. SDS-PAGE also revealed a single protein band. The native molecular mass determined by gel filtration was 33 kDa. The sub-unit molecular mass determined by SDS-PAGE was 33 kDa, suggesting that enzyme is a monomeric protein. The A_{280}/A_{260} ratio was 1.5, clearly indicating that the enzyme is free from bound nucleotides. When the enzyme was stored at 4^{o}C in absence of any additive, $t_{1/2}$ was 90 days. However, when the enzyme was stored at 4^{o}C in the presence of acetaminophen, $t_{1/2}$ increased to 121 days. The K_m and V_{max} values are 0.5 mg/ml and 603 µmol/min/mg, respectively. The k_{cat} was 335 s^{-1}. From the Arrhenius plot, the activation energy was calculated to be 6.9 kcal/mol. When the enzyme was dialyzed against EDTA (1 mM), negligible loss in activity was observed suggesting that there is no absolute requirement of a divalent cation. However, divalent cations namely, Co^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+} at 1mM concentration, activated the enzyme activity. Mn^{2+} inhibited the enzyme activity at the same concentration. NH_4^+ showed activation of the enzyme activity, Li^+ and K^+ showed inhibition. Na^+ had no effect on the enzyme activity. NAD^+ activated the enzyme activity at 1 mM concentration while ATP, ADP, NADH inhibited the enzyme activity. The latter inhibitors at 3 mM concentration showed non-competitive inhibition. The K_i values of NADH, ATP and ADP were 3.96 mM, 2.0 mM and 4.48 mM, respectively. At 5 mM concentration, glycine, fructose1,6-bisphosphate and phenylalanine brought activation to the enzyme 4.9, 2.15 and 2.03 fold, respectively with respect to the control. Dicarboxylic acids like glutamic acid, oxalic acid, oxaloacetic acid and fumaric acid inhibited the enzyme activity. In the presence of citrate and urea, complete loss in the enzyme activity was observed. Bovine serum albumin (0.4 mg) and polyethylene glycol (0.4%) brought a 1.9 and 1.4 fold activation with respect to alpha amylase activity.

Keywords: alpha amylase purification; kinetics; storage and stability; metabolites.

Introduction

Amylases belong to the class hydrolases that hydrolyze O-linked glycosidic linkages of soluble starch (amylose). Alpha amylase (EC 3.2.1.1; 1,4-α-D-Glucan glucanohydrolase) acts on (α1→4) linkages in starch and catalyzes the hydrolysis in a random manner to generate reducing groups in the α-configuration. Alpha amylase finds extensive applications in industries like food (to prepare syrups and bread), brewing (to split starch to malt), detergents (to remove resistant starch residues on clothes), paper (to degrade starch and reduce viscosity), textile (to soften starched clothes) and dyeing (to de-starch starched clothes). The enzyme is important from clinical point of view to diagnose pancreatitis.

Alpha amylase has mostly been studied from microbes (Nishimura *et al*. 1994; Planchot and Colonna, 1995; Das *et al*. 2004; Noudari *et al*. 2010; Sidkey *et al*. 2011) and pancreas of human beings, hog, rat, crab and ostrich (Thoma *et al*. 1971; Oosthuizen *et al*. 1994; Hsieh *et al*. 2008). The enzyme has been purified from few plants like

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barley, sorghum, soybeans, broad beans, wheat (Thoma *et al.* 1971), wheat germ (Sharma *et al.* 2000), maize (Warner and Knutson, 1991; Warner *et al.* 1991), poplar leaves (Witt and Sauter, 1996a), potato (Witt and Sauter, 1996b), tulip (Ranwala and Miller, 2000), finger millet (Nirmala and Muralikrishna, 2003), mung bean (Tripathi *et al.* 2007), Korean pine seed (Azad *et al.* 2009), and soybean (Kumari *et al.* 2010). Partial purification of alpha amylases has also been reported (Beleia and Marston, 1981; Mohamed *et al.* 2009).

Since there are not many reports on plant alpha amylase, the present study has been taken up to get a better insight on this enzyme. The seeds of pearl millet (*Pennisetum typhoides*) were selected for extraction and purification of the enzyme. Pearl millet belongs to the family Poaceae. It is grown under conditions of high temperature and low rainfall. In the present paper we describe the purification, physico-chemical characterization, kinetic properties and role of metal ions and metabolites on alpha amylase from pearl millet (*Pennisetum typhoides*). Its interesting characteristics could probably be exploited for industrial applications.

Materials and Methods

Molecular mass kit of standard proteins was procured from Sigma Aldrich, USA and Bangalore Genei, India). Sepharose 6B, carboxymethyl cellulose, Coomassie Brilliant Blue R-250, acetylsalicylic acid, acetaminophen and ibuprofen were from Sigma Aldrich, USA. All other chemicals were of AR grade procured from SRL, Sd Fine, HiMedia etc. Pearl millet (*Pennisetum typhoides*) seeds were purchased from local market. All solutions were prepared in de-ionized water from Milli-Q system (Millipore, USA).

Enzyme activity assay: The soluble starch solution (1%) was prepared by suspending 0.5 g of starch in 50 ml of assay buffer (50 mM imidazole buffer, pH 7.0) and subjecting to boiling in water bath for 90 s. DNS (3,5-dinitrosalicylic acid) reagent was prepared by suspending 0.5 g of DNS in 25 ml of de-ionized water. To this, 15 g of sodium potassium tartrate was added slowly with stirring. Further, 10 ml of 2N sodium hydroxide was added and final volume was raised to 50 ml with de-ionized water.

A standard solution of maltose (5.0 mM) was prepared in de-ionized water. It was used for calibration with DNS reagent. Absorbance was recorded at 540 nm using Varian 50 Spectrophotometer. The reducing sugar reduces 3,5-dintrosalicylic acid to 3-amino-5-nitro-salicylic acid. A calibration curve was obtained by plotting A_{540} versus maltose concentration in µmol (Data not shown).

The alpha amylase activity was assayed by discontinuous procedure using 3,5-dinitrosalicylate (DNS) reagent as described by Bernfeld (1955). The 1.0 ml assay solution contained 0.5 ml of 1% soluble starch and 0.4 ml assay buffer maintained at 37°C. The reaction was initiated by adding an aliquot of 0.1 ml enzyme solution and incubated for 3 min. The reaction was stopped by adding 1.0 ml DNS reagent, and tubes were placed in boiling water bath for 5 min. Tubes were brought to room temperature and the volume was raised to 10.0 ml with de-ionized water. The blank contained 0.5ml of assay buffer and 0.5 ml of 1% soluble starch solution. One unit of α-amylase was defined as the amount of enzyme releasing one µmol maltose equivalent per minute under the assay conditions.

Protein estimation: Estimation of protein was done by Bradford's method (Bradford, 1976) with some modifications.

Purification of alpha amylase from pearl millet seeds: Pearl millet (*Pennisetum typhoides*) seeds were procured from local market and were washed thoroughly under tap water. The seeds were soaked in de-ionized water at 25°C in a seed germinator. The 18h imbibed seeds were collected (50g). All the following operations were carried out at 0 to 4 °C unless stated otherwise.

(a) *Extraction of enzyme*: The imbibed seeds (50g) were homogenized in 75 ml pre-cooled extraction buffer (50 mM imidazole buffer, pH 7.0 containing 1 mM $CaCl_2$) using kitchen blender giving one burst of 45 s at slow speed. The suspension obtained was squeezed through four layers of muslin cloth and centrifuged in Sigma refrigerated centrifuge at 20,000 rpm (37565x*g*, 25 min). The volume of clear supernatant obtained was recorded. It has been referred to as crude extract. The enzyme units and protein content were determined. The specific activity value was

calculated. The following steps were used to purify the enzyme:

(i) *pH Change step*: The crude extract (pH 7.0) was subjected to a change in pH (pH 6.0) by addition of pre-cooled 0.1 N acetic acid drop by drop with stirring under cold conditions. The extract obtained was centrifuged at 20,000 rpm for 10 min. The pH of clear supernatant obtained was restored to pH 7.0 by addition of pre-cooled 1.0 N NaOH. The volume was recorded and enzyme activity and protein concentration were determined.

(ii) *Lyophilization*: The extract obtained was kept at -80 °C overnight in ultra-freeze and was subjected to lyophilization at -55 °C. The volume of the extract was reduced to almost half of the original extract. It was then centrifuged at 20,000 rpm for 20 min. at 4 °C. The pellet was discarded and the supernatant was used in further steps. The enzyme activity and protein concentration was determined.

(iii) *Polyethylene glycol (PEG) precipitation*: A 50% PEG 6000 solution (w/v) was prepared in assay buffer. The enzyme extract obtained above, was brought to 3% saturation and stirred for 90 min. The suspension obtained was centrifuged at 4 °C for 20 min at 20,000rpm. The pellet was discarded and supernatant was brought to 15% saturation by slow addition of PEG solution. It was stirred for 2h and centrifuged at 20,000 rpm for 20 min. The supernatant was discarded and the pellet obtained was dissolved in minimum volume of extraction buffer. The enzyme activity and protein concentration was determined.

(iv) *Sepharose 6B gel filtration*: In a beaker, 20 ml sepharose 6B was taken and washed with assay buffer four to five times so that ethanol goes off in which it was originally stored. The washed matrix was then placed in an incubator at 30°C for 15h. The activated matrix was then placed in a 1x50cm long column and packed. The column bed was 1x40 cm. The column was washed with several volumes of 25 mM imidazole buffer, pH 7.0 till the pH of filtrate was 7.0.

The enzyme obtained after PEG precipitation step was applied on the column bed. Once it percolated, the enzyme was eluted with 25 mM imidazole buffer, pH 7.0 containing 10 mM β-cyclodextrin (column buffer I). The flow rate of the column was maintained at 15 ml per h with peristaltic pump. Different fractions of 1.0 ml were collected. Each fraction was tested for enzyme activity and protein content. The fractions showing enzyme activity were pooled. The value of specific activity was computed.

(v) *Carboxy-methyl cellulose column chromatography*: Carboxy-methyl cellulose matrix was washed according to Peterson & Sober (1962) and finally stored in equilibration buffer (20 mM imidazole buffer, pH 6.5). A column bed of 1.0x20cm dimension was prepared. The enzyme was applied on top of the column bed and washed with 20 mM imidazole buffer, pH 6.5 (column buffer II). Different fractions of 1.0 ml were collected and tested for enzyme activity and protein content. Once, the washings were free from protein, the elution was done using a gradient of 50 mM imidazole buffer, pH 7.0 and 300 mM KCl (elution buffer). The enzyme activity and protein were tested in all the fractions.

Polyacrylamide gel electrophoresis (PAGE): Native-PAGE was performed according to Ornstein and Davis (1964) with some modifications. SDS PAGE was performed according to Laemmli (1970).

Activity staining: It was performed after carrying out native PAGE. In one set, gels were stained by Coomassie Brilliant Blue as described previously, while other set is activity stained. Reagents required for performing activity staining are: 0.2% iodine, 2% potassium iodide and 1% soluble starch solution prepared in assay buffer in addition to a native PAGE gel. The native PAGE gel with the single enzyme band retrieved from electrophoresis was taken in a clean petridish. To this, 8 ml of 1% starch solution was added gently and then was incubated for 15 min. at 35°C in the incubator, followed by addition of iodine reagent (0.2 g iodine and 2 g KI in 100 ml dissolved in de-ionized water). The gel was submerged in the iodine reagent and incubated at room temperature for 10 min. The iodine starch mixture was then drained out from the petridish. The gel was subsequently photographed.

Molecular mass determination: The mobility of the purified protein was compared with those of

the following molecular mass standard markers: lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa) and BSA (66 kDa). Standard molecular mass marker sample was applied in the well adjacent to the well where purified protein sample was applied. The gel was run at 150 V and was complete in 50 min. The gel was retrieved, stained and bands were resolved. The molecular mass was analyzed with the help of Kodak Digital Science 1 D software. A plot of log molecular mass versus relative mobility was plotted to determine molecular mass of the purified protein.

Gel-filtration using Sepharose 6B for determination of native molecular mass of the purified enzyme was performed according to the protocol given in the Sigma-Aldrich technical bulletin catalog number MWGF200. A column measuring 90 cmx1.6 cm was packed with Sepharose 6B to a total bed volume of 180 ml. The column was equilibrated with a 50mM Tris-HCl buffer pH 7.5 along with 100 mM KCl as the equilibrating buffer. The flow rate was maintained at 20 ml/h. The marker proteins obtained from this Sigma Kit was applied separately on the column with a total volume of 1.0ml each and the concentration as recommended in the bulletin. The kit contained different marker proteins with a range from 12.4 kDa to 200 kDa. The protein samples were cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150kDa) and β-amylase (200 kDa). The elution volume (V_e) of all these sample protein markers was recorded. The void volume (V_o) was determined from the elution volume of Blue dextran (2000 kDa).

The purified alpha amylase enzyme was applied on to this column and fractions having the enzyme activity were collected. The elution volume was recorded. A plot of log Mr versus V_e/V_o was made. The native molecular mass of the enzyme was determined.

Storage and stability: The enzyme was divided into several Eppendorf tubes and stored at 4°C, in the presence of additives (5 mM) like ascorbic acid, acetaminophen, salicylic acid while keeping one set in assay buffer as a control. On different days, aliquots of enzyme were withdrawn from each tube and tested for residual activity. A plot of log % residual activity versus days was plotted and $t_{1/2}$ was determined.

Steady state kinetics – The following assays were performed:

(a) *Effect of pH on rate*: The variation of enzyme activity with pH has been studied using 50 mM imidazole buffers, (pH 5-9) at enzyme saturating concentrations of soluble starch. From the data, a plot of rate of reaction versus pH was plotted to determine pH optima.

(b) *Effect of substrate concentration on rate*: A stock solution of 2% soluble starch was prepared and the rate of reaction was monitored at different concentrations of starch (0.1 mg to 6.0 mg) in the test solution. From this data, Lineweaver Burk plot (1/v versus 1/[starch]) was made to determine values of K_m and V_{max}. From the data, Cornish Bowden plot was also obtained.

(c) *Effect of Temperature*: Effect of temperature on alpha amylase activity has been studied in the range of 30 to 60 °C using 50 mM imidazole buffer, pH 7.0. The reaction mixture was maintained at the particular temperature. Concentration of soluble starch was the same as in the activity assay. From the data, Arrhenius plot was made and energy of activation was determined.

Role of metal ions: The effects of various divalent (calcium chloride, nickel chloride, magnesium chloride, manganese chloride, cobalt chloride) and monovalent (potassium chloride, ammonium chloride, sodium chloride, and lithium chloride) cations have been studied at 1 mM concentration with pearl millet alpha amylase. The enzyme was dialyzed against buffer containing 1 mM EDTA. The dialysis tubing was washed and stored according to Pohl (1990). Solutions of metal ions were prepared in assay buffer. In another experiment, effect of calcium ions was studied at different concentrations. The concentration of starch was same as in the routine activity assay.

Effect of metabolites: A number of metabolites of TCA cycle, nucleotides and amino acids were tested for their effect on alpha amylase activity. Stock solution (50 mM) of each metabolite was prepared in assay buffer. The test solution contained 0.5 ml starch, 0.3 ml assay buffer, 0.1

ml metabolite. The reaction was initiated by addition of 0.1 ml aliquot of enzyme. In a separate experiment, effects of BSA and polyethylene glycol at different concentrations on enzyme activity were tested. K_i values were determined from double reciprocal plots of inhibition of enzyme with respect to starch in the presence of fixed concentration of inhibitors (ATP, ADP and NADH).

Results and Discussion

The wet imbibed (18h) pearl millet (*P. typhoides*) seeds were used for extraction of α-amylase. Several buffers of different pH were tried. Imidazole buffer gave the best result. The maximum specific activity (24 units/mg protein) of alpha amylase was achieved using 50 mM imidazole buffer, pH 7.0 containing 1 mM $CaCl_2$. On further increasing the pH of extraction buffer, there was a fall in specific activity. Therefore the enzyme was routinely extracted with 50 mM imidazole buffer, pH 7.0 containing 1 mM $CaCl_2$. Extraction of alpha amylases from cereals have been carried out using buffers like sodium phosphate and Tris-HCl and pH was above 4.5 to minimize loss of activity due to inactivation (Hill and MacGregor, 1988). For extraction of alpha amylase from mung bean and germinating soybean, sodium acetate buffer, pH 5.5, containing $CaCl_2$ was used (Tripathi *et al*. 2007; Kumari *et al*. 2010).

The results of purification of α-amylase extracted from imbibed seeds of pearl millet are presented in Table 1. Pearl millet seeds (18 h imbibed) were homogenised in pre-cooled extraction buffer with one burst of 45 sec at slow speed. Longer duration of crushing caused an increased extraction of extraneous proteins and shorter crushing periods gave incomplete extraction of alpha amylase. The pH change step and lyophilization steps brought a marginal increase in specific activity. These steps, however, caused removal of extraneous proteins and the resulting solution was clear. These steps were thus included as they facilitated further purification steps. In the next step fractionation was carried out using 50% (w/v) Polyethylene glycol 6000 (PEG) solution. The pellet collected between 3-15% was dissolved in minimum volume of buffer. At this point an overall 4-fold increase in specific activity was achieved with 77% recovery. The enzyme preparation was viscous. In the next step, the above enzyme preparation was applied onto Sepharose 6 B column bed. The column was washed with column buffer I containing β-cyclodextrin and different fractions were collected. The elution profile is shown in Fig. 1. It is evident that, the first protein elution peak had no alpha amylase activity. The enzyme was eluted in the second protein elution peak. This step resulted in ten-fold purification. In the next step, the enzyme collected after Sepharose 6B column was applied onto a CM cellulose column bed. The column bed was washed with several volumes of the column buffer till the washings were free from protein. No alpha amylase activity was detected in these washings. The enzyme activity was eluted by a linear continuous gradient of 0-300 mM KCl. The

Table 1
Purification of Alpha Amylase from Pearl Millet Seeds

Steps	*Total protein (mg)*	*Total enzyme (units)*	*Specific activity (units/mg protein)*	*Recovery (%)*	*Purification (fold)*
Crude extract	56.8	1362.5	24	100	1
pH change step	47.2	1415.75	30	103.9	1.25
Lyophilization	28.4	1193.3	42.0	87.6	1.75
PEG precipitation	10.4	1055.4	101.8	77.4	4.25
Sepharose 6B column chromatography	3.6	918.4	255.1	67.4	10.6
CM Cellulose column chromatography	1.5	859.0	578.8	63.05	24.1

enzyme got eluted as a single peak between 50 mM and 100 mM KCl gradient. The protein and enzyme activity elution profiles are shown in Fig. 2. The step caused 24-fold purification and 63% recovery. The specific activity was 580 Units /mg protein. There was no further increase in specific activity using additional steps.

In the purification protocol, β-cyclodextrin has been used for the elution of enzyme from Sepharose 6B column. Use of β-cyclodextrin has been reported by others also (Tkachuk, 1975; Kumari *et al.* 2010). The specific activity is close to the specific activities of alpha amylases from other sources like *Vicia faba* (Greenwood *et al*. 1965), *Vigna mungo* (Koshiba and Minamikawa, 1981), *V. radiata* (Tripathi *et al.* 2007), *Carthamus tinctorius* (Elarbi *et al*. 2009) and G*lycine max* (Kumari *et al.* 2010). Higher specific activities were obtained with some other plant and microbial alpha amylases (Lefuji *et al*. 1996; Witt and Sauter, 1996b; Nirmala and Muralikrishna, 2003; El-Safey and Ammar, 2004; Das *et al*. 2004; Najafi *et al*. 2005; Sidkey *et al.* 2011). Fold purification of the present enzyme is close to the fold purification of the enzymes obtained from *Aspergillus foetidus* (Michelna and Castillo, 1984), malted finger millet (Nirmala and Muralikrishna, 2003), apple (Kanwal *et al*. 2004) and *Nocardiopsis* 7326 (Zhang and Zeng, 2007). In some cases very high fold purification has been obtained (Tripathi *et al.* 2007; Kumari *et al*. 2010). There are reports on purification of alpha amylase using magnetic alginate beads (Teotia and Gupta, 2001) and super paramagnetic particles (Lin *et al*. 2009). The current report of alpha amylase purification is thus simple and yield, fold purification and specific activity are high enough for practical applications.

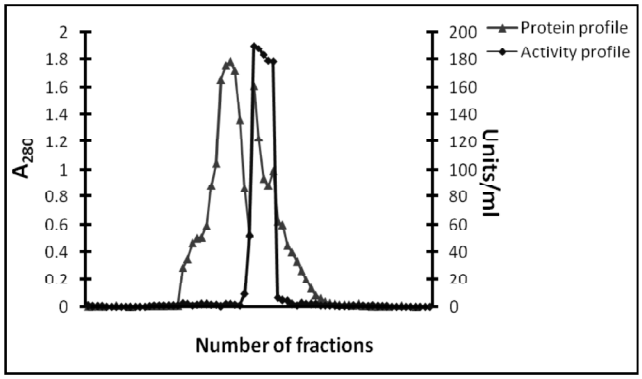

Figure 1: **Sepharose 6B gel filtration column chromatography: Protein (▲) and enzyme activity (♦) elution profile. An activated sepharose 6B matrix was packed in a column to a bed size of 1x40cm. α-amylase obtained from the PEG step was applied to this column and washed several times with 25 mM imidazole buffer, pH 7.0 and further eluted with 25 mM imidazole buffer, pH 7.0 containing 10 mM β-cyclodextrin (Column buffer I). The flow rate of the column was maintained 15 ml per h. Different fractions of 1.0 ml were collected. Each fraction was tested for enzyme activity and protein**

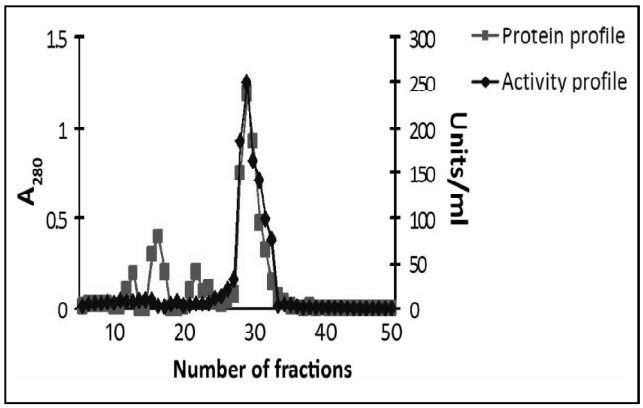

Figure 2: **CM-cellulose column chromatography: Protein (■) and activity (♦) elution profile. The enzyme obtained after Sepharose 6B column chromatography was applied onto a CM cellulose column (1x20 cm) and washed with column buffer II. Different fractions of 1.0 ml were collected and tested for enzyme activity and protein content. Once, the washings were free from protein, the elution was done using a gradient of 50 mM imidazole buffer, pH 7.0 and 300 mM KCl (elution buffer). The enzyme activity and protein were tested in all the fractions**

The native-PAGE shows a single protein band of the native purified enzyme suggesting that enzyme is homogeneous (data not shown). In another set, the electrophoretogram was not stained with Coomassie Brilliant blue but activity stained. A corresponding band is observed by activity staining using iodine reagent (data not shown). This suggests that the protein band in native-PAGE is alpha amylase. PAGE has been extensively used to determine purity of amylases from cereals like wheat (Kruger and Tkachuk, 1969; Merchylo *et al.* 1976; Warchaleswiski and Tkachuk, 1978) and barley (Bilderback, 1974). Alpha amylase from 18 h imbibed seeds of *P. typhoides* on Native PAGE reveals a single protein band when stained with Coomassie Brilliant Blue, suggesting the enzyme to be homogeneous. Other researchers have used activity staining as well (Burgess-Cassler and Imam, 1991; Ramchandran *et al*. 2004; Tripathi *et al*. 2007; Kumari *et al*. 2010 and Noudari *et al*. 2010).

The SDS-PAGE mobility of alpha amylase is shown in Fig. 3a. A single band was observed indicating homogeneity. The relative mobility of the standard molecular mass marker proteins is shown in the adjacent lane. The relative mobility of alpha amylase is compared with the standard molecular mass markers. The plot of log molecular mass versus relative mobility is shown in Fig. 3b. The sub-unit molecular mass of alpha amylase was determined and found to be 33 kDa.

The native molecular mass of alpha amylase from pearl millet was determined by gel-filtration to confirm SDS-PAGE results as well as to investigate the possibility of oligomerization. The Fig. 4 shows a plot of log molecular mass versus V_e/V_o. The elution volume of alpha amylase was measured and the molecular mass of alpha amylase was determined from the plot to be 33 kDa.

The molecular mass of the alpha amylase in the present study was determined by gel-filtration (33 kDa) and SDS PAGE (33 kDa) using standard marker proteins. The results suggest that the enzyme is a monomeric protein. The molecular mass of *P. typhoides* α-amylase is thus close to some other α-amylases (Tkachuk and Kruger, 1974; Fernandez-Tarrago and Nicolas, 1981; Koshiba and Minamikawa, 1981; Moranelli *et al*. 1987; Beers and Duke, 1990; Witt and Sauter, 1996a,b; Nirmala and Muralikrishna, 2003; Mar *et al*. 2003; Noman *et al*. 2006; Tripathi *et al*. 2007; Elarbi *et al*. 2009; Mohamed, 2010).

The spectrum of the enzyme in the ultra-violet region was recorded (data not shown). The maximum absorbance was observed at 280 nm. The A_{280}/A_{260} ratio was found to be 1.5 suggesting that the enzyme is free from any bound nucleotides.

The plot of log % residual activity versus number of days for storage of alpha amylase at 4°C in the presence and absence of acetaminophen is shown in Fig. 5a. It shows linear first order kinetics. When the enzyme was stored at 4°C in the absence of any additive, the $t_{1/2}$ was 90 days. In the presence of acetaminophen (5 mM) the $t_{1/2}$ increased to 121 days. The values of $k_{inactivation}$ are 0.0077 day^{-1} (control) and 0.0057 day^{-1} (acetaminophen), respectively. The effect of other additives like salicylic acid and ascorbic acid are shown in Fig. 5b. Again there is a single exponential decay showing first order kinetics. The per cent residual activity at 100 days in presence of ascorbic acid (87.6%) and salicylic acid (83.0%) is significantly higher than in control (47.2%). Alpha amylase from *Pennisetum typhoides* gave better activity retention when stored in the

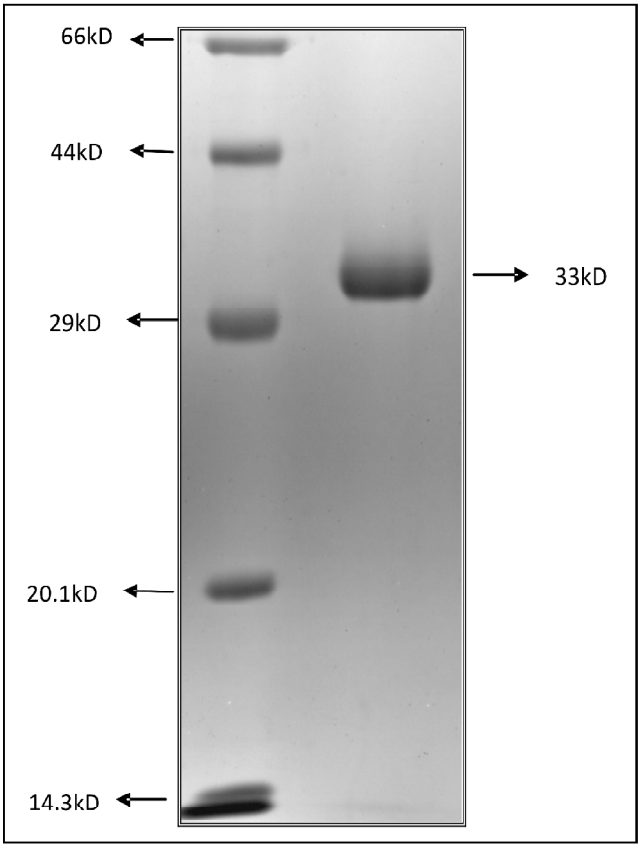

Figure 3a: **Photograph of SDS-PAGE of alpha amylase and mobility of standard molecular mass marker proteins. A routine SDS-PAGE was performed according to Laemmli (1970) with slight modifications along with standard marker proteins (14.3 – 66 kDa)**

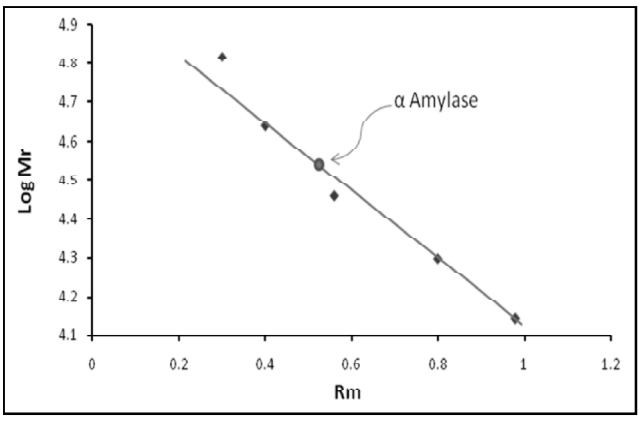

Figure 3b: **Sub unit molecular mass determination of α-amylase: Plot of log molecular mass versus relative mobility**

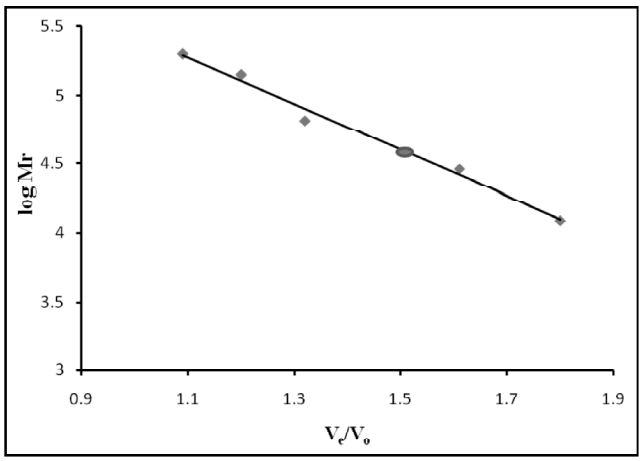

Figure 4: **Molecular mass determination of α-amylase by gel filtration: Plot of log molecular mass versus Ve/Vo. A column measuring 90cmx1.6cm was packed with Sepharose 6B to a total bed volume of 180ml. The column was equilibrated with a 50mM Tris-HCl buffer pH 7.5 along with 100 mM KCl as the equilibrating buffer. The flow rate was maintained at 20ml/h. The marker proteins obtained from the Sigma. The elution volume (V_e) of all the sample protein markers was recorded. The Void volume (V_o) was determined by the elution volume of Blue dextran (2000 kDa)**

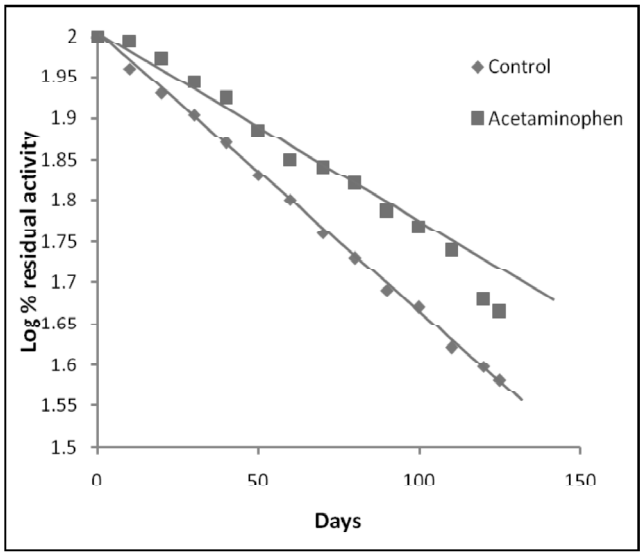

Figure 5a: **Storage stability of alpha amylase at 4°C in the presence of acetaminophen (5 mM) (■). The purified α-amylase was stored in the assay buffer (control) (♦) at 4p C and in another set, it was stored in 5mM Acetaminophen prepared in assay buffer, at 4p C and enzyme activity was assayed at different interval of days**

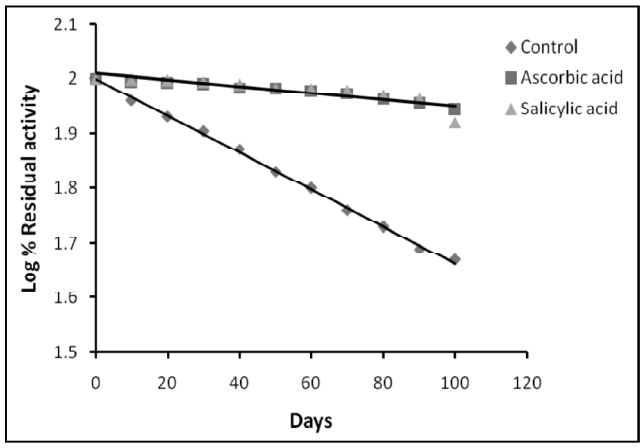

Figure 5b: **Storage stability of alpha amylase at 4°C in the presence of ascorbic acid (5 mM) (■) and salicylic acid (5 mM) (▲). The purified α-amylase was stored in the assay buffer (control) (♦) at 4°C. Enzyme activity was assayed at different interval of days**

presence of additives like acetaminophen, salicylic acid and ascorbic acid at 4°C. The use of acetaminophen, salicylic acid and ascorbic acid has not been reported in previous studies on storage of alpha amylase and offers new options for enhancing storage capability of alpha amylase by solvent engineering. Kapoor and Kapoor (1990) reported the storage of seeds of *Pennisetum americanum* in presence of ascorbic acid. Acetaminophen is one of the NSAID commonly used to bring the body temperature down against fever. The effect of different starches on properties of paracetamol tablets have been reported (Dare *et al.* 2006). When plants are treated with acetaminophen, there is formation of acetaminophen-glucoside and acetaminophen-glutathione conjugates for detoxification (Huber *et al.* 2009). Salicylic acid is known to be a plant hormone (Raskin, 1992). Its effect has been studied on vegetative growth characters yield and grain biochemical components of *Pennisetum typhoides* at elongation and milky stages (Mathur and Vyas, 2007).

The variation of activity with pH has been studied using 50 mM imidazole buffers (pH 5.0-9.0) at enzyme saturating concentrations of starch (data not shown). Maximum activity was observed at pH 7.0, like other alpha amylases from *Triticum aestivum* var. Balady AV isoform (Mohamed *et al*. 2009), *B. licheniformis* EMS-6 (Ikram-Ul-Haq *et al*. 2010) and *B. marini* (Ashwini *et al*. 2011). Alpha amylase from apple and *Pachyrhizus erosus* had pH optimum 6.8 and 7.3, respectively (Kanwal *et al*. 2004; Noman *et al*. 2006). Alpha amylase from *Carica papaya* showed pH optimum 9.0 (Annis, 1982).

The rate of alpha amylase catalyzed reaction was studied at different concentrations of starch

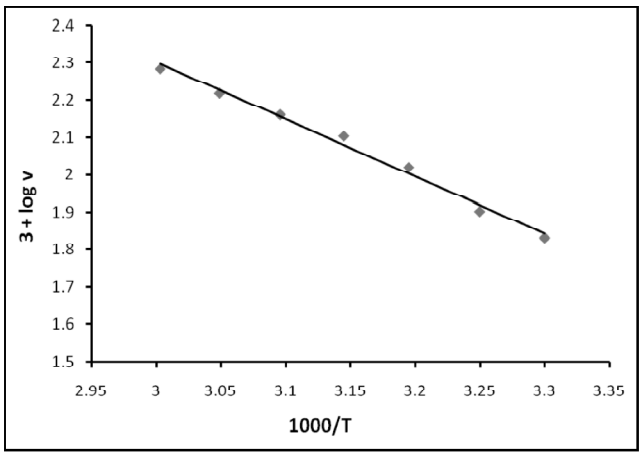

Figure 6: **Arrhenius plot of alpha amylase catalyzed reaction at different temperatures (30-60°C). The temperature of the reaction mixture was varied keeping the fixed concentrations of soluble starch, assay buffer and enzyme**

at pH 7.0. The double reciprocal plot (Lineweaver-Burk plot), yielded K_m and V_{max} values (data not shown) and were found to be 0.5 mg ml^{-1} and 603 µmol $min^{-1}mg^{-1}$protein, respectively, for soluble starch as substrate. A lower K_m value suggests that the enzyme has a very strong affinity for soluble starch. The value is quite close to some other alpha amylases from plant and microbes (Marchylo *et al*. 1976; Khoo *et al*. 1994; Planchot and Colonna, 1995; Glymph *et al*. 1997; Nirmala and Muralikrishna, 2003; Azad *et al*. 2009; Sidkey *et al*. 2011; Kikani and Singh, 2011). In routine alpha amylase activity assay, 1% soluble starch was the substrate, but for determining the K_m and V_{max}, a 2% soluble starch solution was used in the present study. The k_{cat} value for alpha amylase from *P. typhoides* was determined to be $335s^{-1}$. The k_{cat} value is close to the soybean alpha amylase (Kumari *et al*. 2010).

The effect of temperature on rate of alpha amylase catalyzed reaction was studied in the range of 30 °C to 60 °C at the optimum pH (7.0). An Arrhenius plot of the data (log v versus 1/T) is shown in Fig. 6. The energy of activation has been determined from Arrhenius plot. It was found to be 6.9 kcal. mol^{-1}, which is close to some other alpha amylases (Swain and Dekker, 1966; Tripathi *et al*. 2007; Kumari *et al*. 2010).The temperature coefficient, i.e. increase in the rate for a 10°C rise in temperature is found to be 1.4.

The effect of divalent and monovalent cations at 1 mM concentration on EDTA dialyzed alpha-amylase catalyzed reaction rate was studied. The dialysis of the enzyme against EDTA did not bring any loss of activity, suggesting that divalent cations are not absolutely required for activity. The enzyme obtained after dialysis is referred to as control with 100% activity. The results of effect of divalent and monovalent cations on EDTA dialyzed alpha amylase are given in Tables 2a and 2b, respectively. It is evident that Ca^{2+} brought an increase in activity. Calcium ions activate the above enzyme like alpha amylases from other sources (McWethy and Hartman, 1977; Krishnan and Chandra, 1983; Zhang and Zeng, 2007; Hsieh *et al*. 2008; Elarbi *et al*. 2009; Mohamed *et al*. 2009; Femi-Ola and Olowe, 2011). The effect of [Ca^{2+}] in the range of 0.1 mM to 4 mM has been studied on rate of alpha amylase catalyzed reaction (data not shown). At 4 mM [Ca^{2+}], the rate almost doubled. At the same concentration, 1.5 times activation was observed in alpha amylase from *Citrus sinensis* (Mohamed *et al*. 2010). Calcium ions play crucial role in stability of alpha-amylases (Rogers, 1985).

Other divalent cations like Co^{2+}, Ni^{2+} and Mg^{2+} brought activation to alpha amylase activity from *P. typhoides*. These ions also activate other alpha amylases (McWethy and Hartman, 1977; Krishnan and Chandra, 1983; Zhang and Zeng, 2007; Hsieh *et al*. 2008; Femi-Ola and Olowe, 2011). Manganese ions inhibited the enzyme from *P. typhoides* like other alpha amylases from other sources (Krishnan and Chandra, 1983; Abou-Zeid, 1997).

Ammonium ions brought a significant activation of enzyme activity. There are no other reports of effect of NH_4^+ ions on alpha amylase activity, and hence millet alpha amylase may be unique. Na^+ ions had no effect on alpha amylase activity in the present study (Mahadavi *et al.* 2010) while Li^+ and K^+ ions brought inhibition (Ramchandran *et al*. 2005; Noman *et al*. 2006).

Effects of metabolites were tested on alpha amylase activity at close to the K_m values of substrate. In one set of experiments some metabolites were tested for their effects at 1 mM and results are shown in Table 3a. Fructose-6-P and NAD^+ brought activation, ATP and ADP inhibited the activity. PEP on the other hand showed no effect on enzyme activity. In another

set of experiment, some other metabolites were tested at 5 mM and results are shown in Table 3b. Glycine, fructose-1,6-bisphosphate and phenylalanine were strong activators. Serine, glutamine, ascorbic acid and salicylic acid were moderate activators and α-ketoglutarate, pyruvate, cysteine, aspirin, acetaminophen, ibuprofen and were mild activators. Methionine had no effect on activity. Glutamic acid, oxalic acid, NADH, alanine, oxaloacetic acid and fumaric acid inhibited the enzyme activity. In the presence of citrate and urea there was a complete loss of enzyme activity.

Table 2a
Effect of Divalent Cations (1 mM) on the Activity of EDTA Dialyzed Alpha Amylase

Divalent cations	*Activity*
(1 mM)	(%)
Control	100
Co^{2+}	137.2
Ca^{2+}	135.9
Ni^{2+}	127.3
Mg^{2+}	121.4
Mn^{2+}	34.8

Table 2b
Effect of Monovalent Cations (1 mM) on the Activity of EDTA dialyzed Alpha Amylase

Monovalent cations	*Activity*
(1 mM)	(%)
Control	100
NH_4^+	179.2
Na^+	101.8
Li^+	71.6
K^+	61.3

Table 3a
Effect of Metabolites (1 mM) on Alpha Amylase Activity

Metabolite	*Observed activity (% of control)*
Nil (Control)	100
Activators	
NAD^+	142.6
Inhibitors	
ATP	79.8
ADP	72.19
No effects	91-110
PEP	97

Table 3b
Effect of Metabolites (5 mM) on Alpha Amylase Activity

Metabolite	*Observed activity (% of control)*
Nil (Control)	100
Activators	
Glycine	496
Fructose-1,6-bisphosphate	215
Phenylalanine	203
Serine	182
Ascorbic acid	170
Glutamine	152
Salicylic acid	139
α Ketoglutarate	132
Aspirin	132
Acetaminophen	131
Pyruvate	126
Ibuprofen	121
Cysteine	119
Inhibitors	
Glutamic acid	67
Oxalic acid	45
Alanine	38
Oxaloacetate	27
Fumaric acid	26
Citrate	0.0
Urea	0.0
No Effect	
Methionine	99

In a separate experiment, the inhibition of alpha amylase at different starch concentrations in the presence of inhibitors NADH, ATP and ADP at 3 mM concentrations was studied. In every case non-competitive inhibition was observed. The K_i values were determined and results are shown in Table 4.

Table 4
Values of K_i and Apparent V_{max} of the Inhibitors

Inhibitor	*Inhibitor (mM)*	*K_i (mM)*	*Apparent V_{max} (μmol/min/mg)*
NADH	3	3.96	342.5
ATP	3	2.0	238.2
ADP	3	4.48	360.6

The effects of different metabolites have been tested on alpha amylase activity from *P. typhoides*. The results are contrasting when tested for NAD^+

and NADH. In the presence of NAD^+ activation has been observed while inhibition in the presence of NADH. It is possible that both may bind to some other site to bring about effects. The non-competitive inhibition in presence of NADH confirms its binding to a different site. The alpha amylase activity may be governed by concentrations of NAD^+ and NADH. When NAD^+ is more, it activates breakdown of starch into sugar to push later for their conversion to pyruvate. On the other hand, when NADH is more, glycolytic pathway is checked and so the breakdown of starch. In plants, however, starch is converted to glucose-1 P in plastid in the presence of phosphorylase (Plaxton, 1996). This shows how alpha amylase might be regulated *in vivo*.

Fructose 1,6-bisP is known to be an activator of glycolytic pathway. In the present study, its effect was tested on alpha amylase activity and was found to activate the enzyme activity 2.15 fold. In the presence of higher fructose-1,6-bisP level, starch hydrolysis to glucose may be promoted to enrich the raw material for the glycolytic pathway. Fructose-1,6-bisP is hydrolyzed in presence of fructose-1,6-bisphosphatase to fructose-6P in Calvin cycle. There are also no other reports on use of glycolytic intermediates. Glycine at 5 mM concentration brought about a 4.9 - fold activation. Glycine is involved in the synthesis of chlorophyll and is also formed by transamination of glyoxylate in photorespiration. Glycine content is high in some of the alpha amylases (Planchot, and Colonna, 1995; Nirmala and Muralikrishna, 2003). Higher glycine concentration may also stabilize the enzyme structure.

Dicarboxylic and tricarboxylic acids such as citric acid, oxalic acid and oxaloacetic acid brought inhibition to the alpha amylase activity in the present study like alpha amylase from *Rhizopertha dominica* (Priya *et al*. 2010). It may be due to chelation of divalent metal ion. Salicylic acid is a plant hormone and has a role in the signaling pathway in plants (Raskin, 1992; Klessig and Malamy, 1994) and activates *P. typhoides* alpha amylase. Activation of *P. typhoides* alpha amylase has been observed in presence of acetaminophen, ibuprofen and aspirin and ascorbic acid. The presence of the above also helped in the storage of enzyme. There are however no reports of effects of acetaminophen, ibuprofen, aspirin and ascorbic acid on alpha amylase activity.

Bovine serum albumin (0.4 mg) and polyethylene glycol (0.4%) brought 1.9 and 1.4 fold activation, respectively, to activity of alpha-amylase from *P. typhoides*. Alpha amylase from porcine pancreas and *Bacillus amyloliquefaciens* also showed activation in the presence of polyethylene glycol (Mukerjea *et al*. 2006). Polyethylene glycol and bovine serum albumin also brought activation to pyruvate kinase by stabilizing the enzyme (Podesta and Plaxton, 1993). Alpha amylase from pearl millet thus offers a suitable enzyme for industrial applications and our study provides scope for solvent engineering in similar amylases.

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Abbreviations

PAGE, Polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; NSAID, Non-steroidal anti inflammatory drugs; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PEP, phospho*enol*pyruvate; NADH, nicotinamide adenine dinucleotide (reduced); NAD^+, nicotinamide adenine dinucleotide (oxidized)

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