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CHARACTERIZATION OF ALPHA-AMYLASE FROM THE SEEDS OF *Mucuna pruriens*

K S Chandrashekharaiah*[1], Preethi[1], Shalini[1], V Krishna Murthy[1], M Narayanaswamy [2], K R Siddalinga Murthy[3], N Ramachandra Swamy[3]

[1]Department of Biotechnology, PES Institute of Technology, Bangalore-560085.
[2]Department of Molecular Biology, Bangalore University, Bangalore-560056.
[3]Department of Biochemistry, Bangalore University, Bangalore-560001.

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ABSTRACT

Amylases are hydrolytic enzymes which are widely distributed in nature, animals, plants and microorganisms. Amylases are of great significance in present-day biotechnology. In present study, amylases are isolated from the soaked seeds of *Mucuna pruriens* under extreme acidic conditions. Conventional protein purification techniques such as salt fractionation, ion exchange chromatography on CM-cellulose and sephadex G-75 was employed for the purification of amylase from the seeds of *Mucuna pruriens*. The amylase activity was eluted in one peak. The specific activity and yield of the purified amylase was 6.25 and 29.99, respectively. Native PAGE, SDS-PAGE and gel electrofocussing were employed to establish homogeneity of the purified amylase. SDS-PAGE and gel-filtration chromatography on sephadex G-75 was used to determine the molecular weight of the purified amylase. The purified amylase was nearly homogenous and its molecular weight was found to be 78.4 kDa. The optimum pH and temperature of the purified amylase were 7.0 and 50^{o}C, respectively. The isolectric pH of the purified amylase was 7.2 and the activity was linear up to 60 minutes.

* Corresponding author

E-mail: kschandraks@gmail.com (K S Chandrashekharaiah)

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1 Introduction

Alpha-amylases (α-1,4 glucan –glucanhydrolase, EC 3.2.1.1) are hydrolytic enzymes catalyze the hydrolysis of α -1,4 glycosidic linkages and randomly producing oligosaccharides and monosaccharides (Omemuet et al., 2005; Bhanja et al., 2007; Laman et al., 2009). Most of the isolated α-amylases are metal activated enzymes, which require calcium ions for their catalytic activity and structural stability. Amylases are one of the most important and widely used enzymes in pharmaceutical, fine chemical, detergent, textile, paper, food, baking, brewing industries as well as in clinical, medicinal and analytical chemistry. (Carlsen et al., 1996; Ramachandran et al., 2004; Kathiresan & Manivanan, 2006; Gupta et al., 2008).

The genus *Mucuna* belongs to the family Fabaceae (leguminaceae) which contains annual and perennial legumes of pantropical distribution. The genus *Mucuna* and its varieties/accessions are rich in crude protein (24 and 31.44%) which surpasses many wild and edible legumes. Traditionally, the seeds of *Mucuna* are staple food for many tribal people of Asia and Africa. The immature pods and leaves serve as vegetables, while seeds as condiment and main dish by ethnic groups in Nigeria (Adebowale et al., 2005a). Although, the *Mucuna* seeds are good sources of proteins, also contain antinutritional factors like trypsin and chymotrypsin inhibitors. These inhibitors reduce the quality of proteins and impair the digestion of proteins by binding to protein digesting enzymes, trypsin and chymotrypsin. Further, the presence of L-DOPA in the seeds of *Mucuna* increased its demand in the pharmaceutical industries. *Mucuna* being an important medicinal leguminous plant and as a potential source of stable amylase, the present study was undertaken to identify and study the physico – chemical properties of amylase from the seeds of *Mucuna* (Chandrashekharaiah et al., 2011).

2 Materials and Methods

2.1 Mucuna pruriens seeds:

Mucuna pruriens seeds were obtained from local markets of Bangalore, India.

2.2 Preparation of the crude enzyme

The seeds of *Mucuna pruriens* was soaked in distilled water for 24 hrs and seed coat was removed. The enzyme extract is then prepared by blending seeds in a mixer in the ratio of 1:10 using 0.1M HCl containing 0.1M NaCl and 1%PVP. It is then stirred for 3hrs using magnetic stirrer. The suspension is then centrifuged at 12,000rpm for 15 minutes and the supernatant was collected for further assay (Giri & Kachole, 1996).

2.3 Amylase assay

Amylase activity was determined according to the method of Bernfeld (1955). The enzyme assay mixture contained 0.5 ml of enzyme extract, 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.0 an d 1.0 ml of 1% starch. The reaction mixture was incubated for 15 min at room temperature followed by arresting the reaction by the addition of 1.0 ml of dinitrosalicilic acid reagent (DNS reagent). The optical density was read at 540 nm against reagent blank after addition of 3.0 ml of distilled water.

2.4 Protein assay:

The protein content in the enzyme extracts were determined by the method of Lowry et al., (1951)

2.5 Electrophoresis

Slab gel electrophoresis was carried out at pH 4.3 essentially according to the method of Reisfield et al., (1962). A discontinuous gel system consisting of 7.5% separating and 5% stacking gel were used. The separating gel system consisted of Acrylamide and bis acrylamide solution (4ml), separating buffer (5.2 ml), ammonium persulphate (25µl), riboflavin (0.3 ml) and distilled water (6.475ml). This mixture was poured into a clean and dry glass plates and allowed to photopolymerize under a fluorescent lamp for 30 min. After polymerization, the stacking gel solution was poured. The stacking gel consists of acrylamide and bis acrylamide solution (0.67ml), seperating buffer(1.25 ml), ammonium persulphate (25µl), riboflavin(0.3 ml) and Distilled water(2.775ml). The stacking gel solution was poured onto the top of the separating gel and allowed to photopolymerize under a fluorescent lamp for 30 minutes. Then the gels were placed in the elctrophoretic chamber. The electrode chambers was filled with electrode buffer of pH 4.5 (3.12 g β-alanine + 0.8 ml glacial acetic acid diluted to 600 ml with distilled water). The samples suitably diluted (100 µg protein) with 20% sucrose containing methyl green, were loaded on to each sample well and subjected to electrophoresis in cold (4^0 C) applying a current of 20 to 25 m A /gel for 5 - 6 hours.

2.6 Ammonium sulphate precipitation

The crude enzyme extract was subjected to ammonium sulphate precipitation. Solid ammonium sulphate was added slowly with constant stirring over magnetic stirrer at 4^0C to obtain 80% saturation. The solution was allowed to stand for 1 hr at 4^0C, and the mixture was then kept inside refrigerator at 4^0C for 30min. The precipitated protein (0– 80%) was recovered from solution by centrifugation at 10,000 rpm for 30 min. The protein pellet dissolved in 10mM Acetate buffer and it was then dialyzed against acetate buffer pH 5.5. The dialyzed 0 – 80% ammonium sulphate fraction was subjected for further purification employing ion exchange chromatography using CM-cellulose.

2.7 CM-cellulose Ion Exchange Chromatography

Ten grams of CM- Cellulose (1.09 m.eq/gm) was suspended in distilled water and kept aside for 2 hours. It was then filtered by suction. The moist cake was sequentially suspended in 0.1 M sodium hydroxide, 0.1 M HCl and 0.1 M NaOH for 15 minutes and fines were decanted followed by washing with distilled water till the pH was neutral. The CM- cellulose was then equilibrated with 0.025 M acetate buffer pH 5.5 for overnight at 4^0C. The slurry of CM- cellulose was then packed into a 2.5 x 22 cm column and equilibrated with three volumes of starting buffer (0.025 M acetate buffer pH 5.5) at a flow rate of 40 ml per hour. The dialyzed 0 – 80% ammonium sulphate fraction (40 ml was loaded onto the column and washed with 200 ml of the starting buffer. The column was then eluted by stepwise increase in ionic strength using starting buffer containing 0.1 M, 0.3 M and 0.5 M NaCl and ml fractions were collected.

2.8 Sephadex G-75 Gel-filtration chromatography

Sephadex G-75 was allowed to swell in excess of distilled water on a boiling water bath for 5 hrs. The gel was decanted to remove fines and then equilibrated with 0.020 M sodium phosphate buffer, pH 7.0. The gel was packed into a column of size 1.0 cm X 140.0 cm under gravity. The column was equilibrated with two bed volumes of 0.020M sodium phosphate buffer, pH 7.0 at a flow rate of 10 ml/hr. The concentrated CM-cellulose fraction I (4 ml) was loaded onto the gel and the proteins were eluted with 0.025M sodium phosphate buffer, pH 7.0 and 2.0 ml fractions were collected. The peak fractions containing amylase activity obtained from sephadex G-75 were collected.

2.9 Gel-electrofocussing

Gel-electrofocussing was performed by the method of Wringley (1969) with 7% polyacrylamide gels. The gel solution prepared by 0.6 acrylamide solution (29.2 gm acrylamide and 0.8 gm N, N-methylene bisacrylamide in 100 ml of water), 0.06 ml of 40% ampholyte carrier (pH 3 to 10 range), 0.12 ml of riboflavin (0.14 mg/ ml), 0.1 ml enzyme (100 µg protein) and 1.62 ml of water. This solution (1.5 ml was poured into glass tubes (0.6 x 8 cm). The lower anode chamber was filled with 0.2% sulphuric acid and the upper cathode chamber with 0.4% ethanolamine. After the run the gels were removed from the tubes and stained for protein using staining solution (0.02% coomassie brilliant blue G-250 (w/v) and 10% trichloroacetic acid) and distained using distilled water.

2.10 Sodium dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out by the method of Smith (1984). The gel solution contained one part of separating gel buffer (45.5 gm of tris buffer and 1 gm of SDS dissolved in 200 ml, pH was adjusted to 8.8 with HCl and made up to 200 ml), five parts of acrylamide-bisacrylamide solution (30.0 gm acrylamide and 0.8 gm of N, N-methylene bis acrylamide), one part of TEMED, one part of ammonium per sulphate (1 mg/ 10 ml) and seven parts of distilled water. After polymerization, the water layer was removed carefully and spacer gel (one part of stacking gel buffer (8.5 gm of tris and 1 gm of SDS dissolved in 100 ml of water, pH was adjusted to 6.8 with HCl and made up to 200 ml), one part of acrylamide-bisacrylamide solution (30.0gm acrylamide and 0.8gm of N, N-methylene bis acrylamide), one part of TEMED, one part of ammonium per sulphate (1 mg/ 10 ml) and two parts of distilled water. This was allowed to polymerize for 30 min.

2.11 Characterization of purified amylase

2.11.1 Determination of molecular weight by SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified amylase was carried out according to the method of Smith (1984). Carbonic anhydrase, bovine serum albumin, cytochrome-c and phosphorylase-b were used as marker proteins. 0.2ml (100 µg) of each marker protein and purified amylase (100 µg each) in 0.05 M sodium phosphate buffer, pH 7.0, were mixed with 2mg SDS and 0.1ml of glycerol containing 3µl of bromophenol blue in one set and in another set 0.2 ml (100 µg each) in 0.05M sodium phosphate buffer, pH 7.0, were mixed with 2mg SDS, 10 µl of 2-mercaptoethanol and 0.1ml of glycerol containing 3 µl of bromophenol blue. The samples were then boiled for 5 mins. Proteins were then electrophorised in slab gels, stained using coomasie blue and distained by using methanol, acetic acid and distilled water in the ratio of 40:10:50.

2.11.2 Determination of Isoelectric point

The IEF for the purified amylases was determined according to the method of Wringley et al., (1969).

2.11.3 Determination of optimum pH and pH stability

The effect of pH on the activity of the purified amylase was studied using the following buffers: 1) Sodium acetate (0.05M, pH 4.0 – 5.0) 2) sodium citrate (0.05M, pH 5.5 -6.0), 3) sodium phosphate (0.05 M, pH 6.5 – 7.5), 4) tris-HCl (0.05M, pH 8.0 – 9.0), 5) acetate buffer (0.05M, pH2-3).The catalytic activity of the enzymes in the above buffers was determined with starch as substrate. The pH stability of the purified amylase was determined by incubating with buffers of different pH (0.2M, pH 4.0 – 10.0) for 24 hours at 4°C. After incubation the pH was readjusted to their optimum pH and assay has been carried out at their optimum temperature for 15min and maltose formed was determined colorimetrically.

2.11.4 Determination of optimum temperature and temperature stability

The effect of temperature on the activity on the purified amylase was studied at different temperatures ranging between (4°C – 80°C).The enzymes were diluted suitably and incubated

with substrate starch at different temperature for 15 min. The amount of maltose released was measured colorimetrically.

3 Results and Discussion

3.1 Purification of α-amylases

The α-amylase from seeds of *M. pruriens* was purified by conventional methods of protein purification. The acidic crude enzyme extract was subjected to ammonium sulphate fractionation, change of pH and by chilled acetone. Considerable loss of amylase activity was observed with change in pH and acetone.

On the other hand, ammonium sulphate fractionation resulted in good yield with an increase in fold purification. Hence, ammonium sulphate precipitation was selected for fractionation of amylases from the crude enzyme extract.

The ammonium sulphate fraction was subjected to ion exchange chromatography on CM-cellulose. The elution profile of CM – cellulose chromatography is shown in Fig.1. Four peaks of proteins were eluted and were designated as fraction I, fraction II and fraction III and fraction IV. Fraction I was not adsorbed onto the column and hence eluted out with the starting buffer. Fraction II, III and III were eluted by 0.1, 0.3 and 0.5 M sodium chloride, respectively. The fraction IV containing appreciable amount of amylase activity were pooled separately, dialyzed against acetate buffer and concentrated using ammonium sulphate.

The CM-cellulose fraction IV was subjected to gel filtration on sephadex G-75 column. The elution profile of sephadex G-75 chromatography is shown in Figure 2. The protein and the amylase activity were eluted in one peak. A purification profile is shown in the figure 3.1 and figure 3.2. A purification table showing the recovery, fold purification and the specific activity at each stage is given in table 1.

Table 1 Purification of amylases from seeds of *M. pruriens.*

Purification step	Total volume (ml)	Total protein (mg)	Total activity (µ mole/min)	Specific activity	Fold purification	% yield
Crude	175	847	793.3	0.9366	1	100
0-80% ammonium sulphate fractionation	60	739.2	720	0.9741	1.04	90.76
CM- cellulose chromatography (Fraction IV)	70	92.4	466.66	5.050	5.18	64.81
Gel filtration chromatography (Fraction I)	15	72.6	139.99	6.26	7.2	29.99

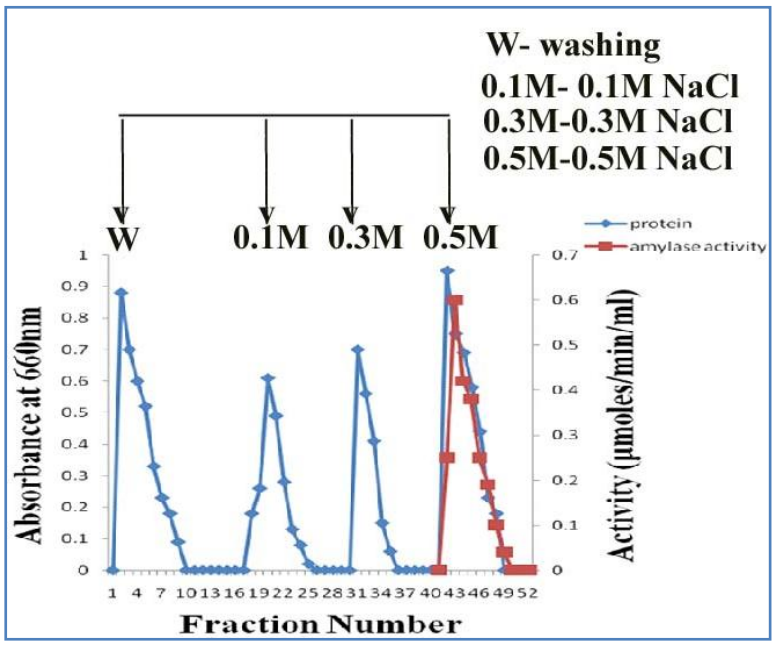

Figure1 Elution profile of amylases of soaked seeds of *M. pruriens* on CM-Cellulose.

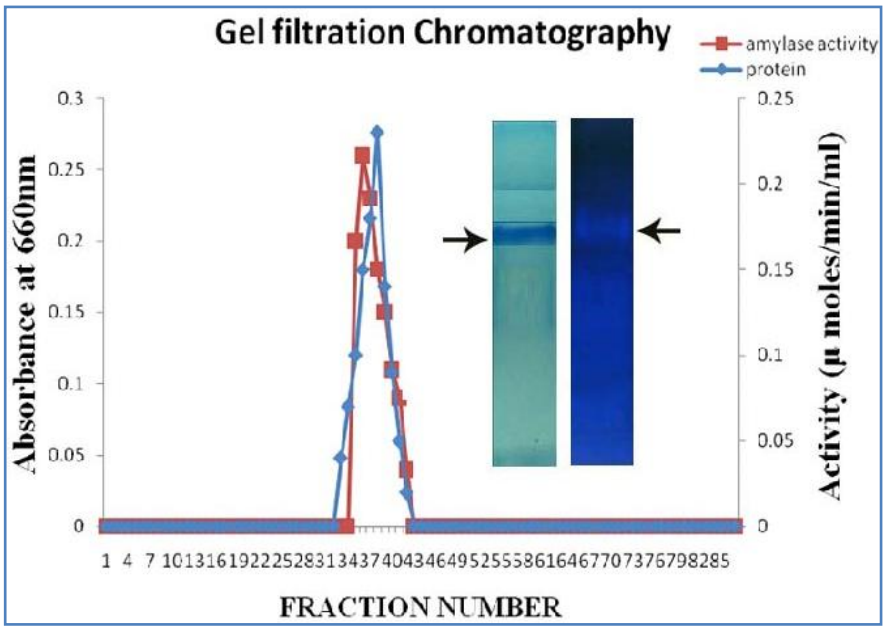

Figure 2 Elution profiles of amylases of soaked seeds of *Mucuna pruriens* on Sephadex G-75.

Thus, in the present investigation, PAGE, IEF and SDS-PAGE have been employed to establish the homogeneity of the *M. pruriens* amylase. Amylases have been purified from various plant sources including Wheat (Machaiah et al., 1984), *Oriza sativa* (Mitsui et al., 2000), *Arabidopsis thaliana* (Sparla et al., 2001) *Phaseolus aconifoliu.* (Nerkar et al., 2011), *Yemini bean* (Mather et al., 2011), *Little millets* (Usha et al., 2011) by employing different biochemical techniques including ammonium sulphate fractionation, ion exchnage chromatography, and gel filteration chromatography.

Three monomeric alpha-amylases (E.C. 3.2.1.1) with a molecular weight of 45.2 kDa from finger millet malt were isolated and purified employing acetone fractionation, anion-exchange chromatography on DEAE-Sephacel and gel-filtration chromatography on Sephacryl S-200 (Nirmala & Muralikrishna, 2003). The binding of these amylases to anion exchange resin indicated the presence of more number of acidic amino acids. In our study, amylase from the seeds of *M. pruriens* was purified by cation exchange chromatography (CM-cellulose). The binding of *M. pruriens* amylase to cation exchange resin indicated the presence of more number of basic amino acids.

3.2 Characterization of partially purified alpha amylase enzymes

The homogeneity of amylase was established by PAGE, IEF and SDS-PAGE. Both SDS- PAGE and IEF showed single protein band and a corresponding amylase band (Figure 3.3 and Figure 4).

SDS-PAGE in the presence and absence of β-mercaptoethanol showed single protein bands, suggesting the monomeric nature of the *Mucuna* amylase enzyme.

3.3 Optimum pH and pH stability

The optimum pH of purified amylase was 7.0 and the enzyme was stable between pH 2 – 10 (Fig. 5a and Fig.5b). The optimum pH for most of the purified α-amylases of animals ranged from 7-9. Among plant α- amylases, it is reported that the optimum pH of 7 was obtained for *Yemini bean* (Mather et al., 2011).

3.4 Optimum temperature

The optimum temperature of the purified amylase of *Mucuna pruriens* was at 50°C (Fig. 5c). The activity increased with increasing temperature to 30 - 50°C followed by a sharp decline to 80 °C where complete inhibition in the activity was found. The purified amylase was found to be optimally active at 50ºC. The activity increased sharply with gradual increase in temperature up to 50ºC while it gradually declined with further rise in temperature, indicating loss in the active conformation of the enzyme.

3.5 Molecular weight determination

The molecular weight of amylase of *M. pruriens* as determined by SDS PAGE was found to be 68.4 kDa both in the presence and absence of 2-mercaptoethanol.

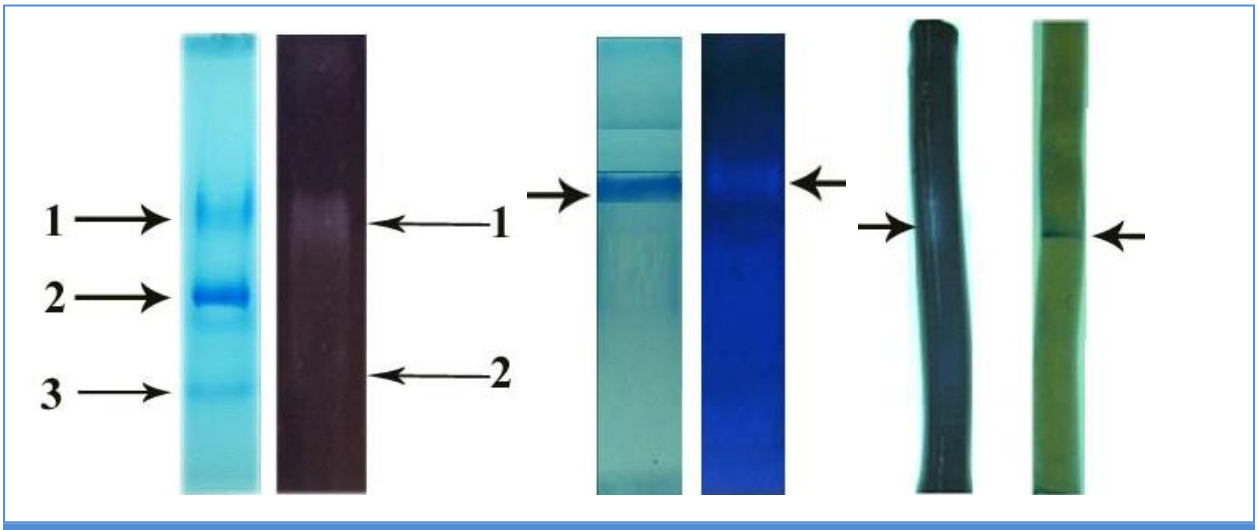

Figure 3(1) Figure 3 (2) Figure 3(3)

Figure 3(1) Native page pattern of protein and amylases of dialyzed sample of *Mucuna pruriens*.
Figure 3(2) Native page pattern of protein and amylases of Gel filtration of *Mucuna pruriens*.
Figure 3(3) Gel electrofocussing pattern of purified pattern of *Mucuna pruriens*.

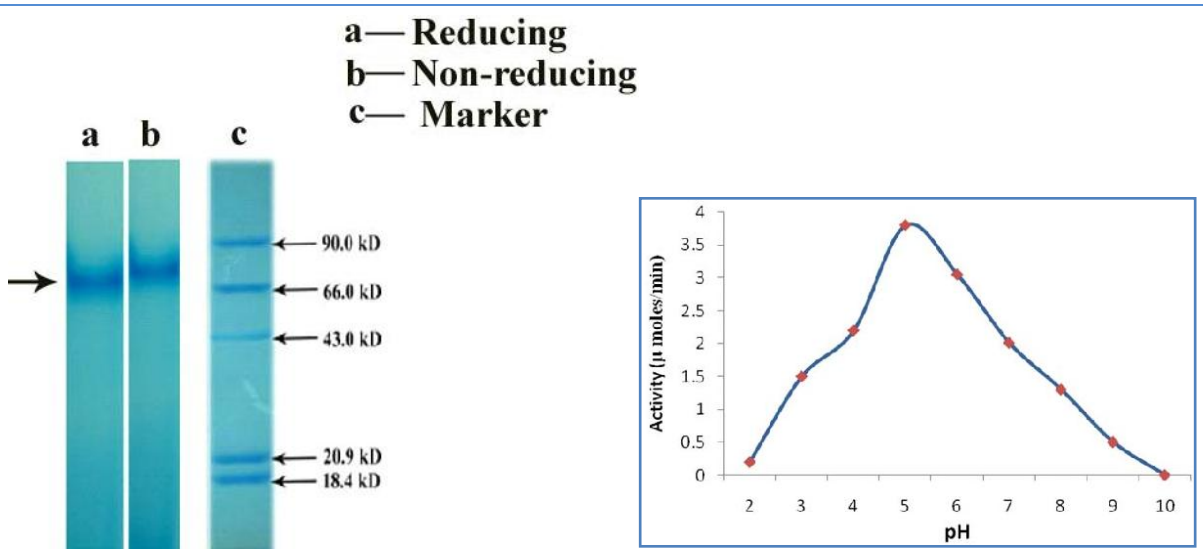

Figure 4(1) Figure 5(a)

Figure 4 SDS-PAGE pattern of standard protein(c) and purified Mucuna seed amylase in the presence (b) and absence (B) of 2-mercaptothanol
Figure 5a Determination of pH stability *of Mucuna pruriens*

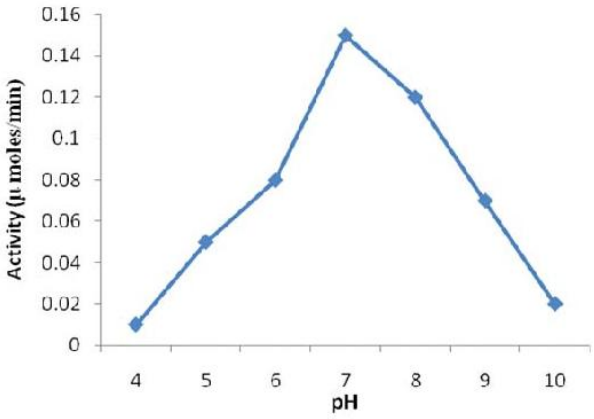

Figure 5(b)

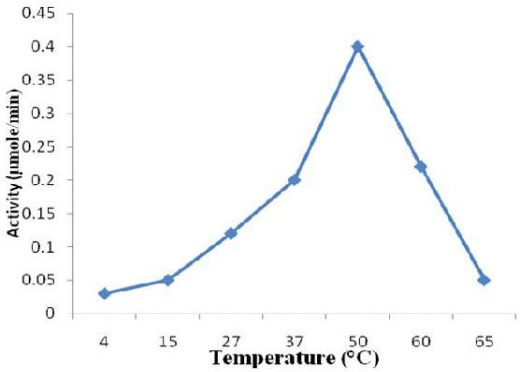

Figure 5(c)

Figure 5b Determination of optimum pH of *Mucuna pruriens.*
Figure 5c Determination of Optimum temperature of *Mucuna pruriens.*

The molecular weight of amylases from *Phaseolus aconifoliu* is found to be 65 kDa (Nerkar et al., 2011) and *Little millets* had a molecular weight of 54.5 KDa.(Usha et al., 2011).This current study indicated that purified amylase from the seeds of *M.pruriens* had a molecular weight similat to amylases from *P. aconifoliu.*

3.5 Isoelectric pH

The Isoelectric pH value of amylase was determined by gel- and it is found to be 7.2. Most of the amylases studied so far have low pI values in both animals and plants and contain large number of acidic amino acids.

The low pI values, the presence of large proportions of acidic amino acids and binding affinity to anion exchanger clearly indicating the acidic nature of amylases in many cases. However this is not in the case of purified *Mucuna* seeds because they have very high pI values and showed affinity binding to cation exchanger. The near basic pI values and binding affinity to cation exchanger probably indicating the presence of large proportions of basic amino acids and hence basic nature of enzymes.

Conclusion

α-amylase has been isolated and purified from the seeds of *Mucuna pruriens* using conventional protein purification techniques such as salt fractionation, CM-cellulose and sephadex G-75 chromatography. The enzyme was isolated in acidic medium. The stability in the extreme acidic pH and temperature indicated that the amylase was both thermo and pH stable. The amylase enzyme from the seeds of Mucuna can be used commercially at extremes of pH and temperature.

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