OPEM
www.opem.org
Oriental Pharmacy and Experimental Medicine 2004 **4(4)**, 227-234

Purification and partial characterization of α-amylase from soybean (*Glycine max*)

Pallavi Tripathi, Alka Dwevedi and Arvind M Kayastha*

School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi-221 005, India

SUMMARY

An α-Amylase was purified to apparent homogeneity from germinating soybean seeds (*Glycine max*). Enzyme showed high specificity for starch. α-Amylase from soybean has optimum pH at 7.6 in the pH range 4.0-10.6. At this pH, the K_m of starch was 2.63 mg/ml and the V_{max} was equal to 52.6 mg/ml/min protein. Optimum temperature of the enzyme was found to be 55℃, Q_{10} equal to 1.85 and energy of activation equal to 12 kcal/mol. Additives like, EDTA reduced the activity of α-amylase whereas PMSF enhanced the activity. α-Amylase was inhibited by several heavy metal ions.

Key words: α-Amylase; Purification; Starch; Soybean; Substrate specificity; Inhibitors

INTRODUCTION

Germination of many seeds, including those of important food grains, requires production of amylases to degrade starch in the seed endosperm for use by the developing plant embryo. A number of investigations relating to the mechanism of α-amylase formation in cereal seeds have been made (Bewley and Black, 1978). In non-endospermic leguminous seeds such as pea, it is also known that α-amylase activity in cotyledons increases during germination with concurrent mobilization of storage starch.

α-Amylases (1,4-α-D-glucan glucanohydrolases; EC 3.2.1.1) catalyse the hydrolysis of internal α-D-1,4 glucosidic linkages in starch (amylose and amylopectin), glycogen and related oligosaccharides. They are monomeric enzymes widely occurring among animals, higher plant, fungi and micro-organisms however; the characteristic of enzymes vary remarkably even from tissue to tissue within a single species (Robert *et al.*, 2002). Amylases has potential applications in various industries *viz.*, in liquefaction of starch pastes in the manufacture of high fructose corn syrup, in brewing industry, as sizing agent, in detergent industry and also used for curing digestive disorders (Hemmingsen, 1980; Jensen and Rough, 1988). The correlation between salivary amylase and stress has been a matter of investigation by various scientific groups (Chatterson *et al.*, 1997; Walsh *et al.*, 1999). To quantitatively evaluate the reactions to stress, a flow-injection-type biosensor system for salivary amylase activity has been described recently (Yamaguchi *et al.*, 2003).

Soybean is economically very important. It is found to be the richest source among various other plant sources. Using this as the source of enzyme, the byproduct produced is having much importance to poultry, fisheries, feed for dairy cattles etc. It has been reported that it is very much beneficial to lactating dairy cows, pig, and chicken due to its easy digestibility. However, no work has been reported

*Correspondence: Dr Arvind M Kayastha, School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi-221 005, India. Tel: +91-542-2368331; Fax: +91-542-2368693 E-mail: kayastha@fastmail.

on α-amylase isolation from soybean. In the present study we report the purification and partial characterization of α-amylase from soybean.

MATERIALS AND METHODS

Plant material

The seeds of soybean (*Glycine max*) were procured from local market. Seeds were washed thoroughly with distilled water and soaked overnight in extraction buffer (0.02 M Tris-HCl, pH 6.5). Seeds were germinated for 60 h at 35℃ on moist sand beds.

Chemicals

All chemical used for PAGE were of Electrophoretic grade; PMSF, DTT, maltose, amylopectin, starch were obtained from Sigma Chemical Co., St. Louis, U.S.A. Sephadex G-75 was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Acetone (HPLC Grade) was purchased from Spectrochem, India. Ammonium sulfate (enzyme grade) was purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used were of analytical reagent grade from Merck. All solutions were prepared by using Millipore water.

Amylase activity assay

Amylase activity was routinely assayed by iodine method however, for specificity studies DNS method was used. (a) Iodine Method: α- amylase was assayed by the iodine method described by Fuwa (1954) with slight modifications. The reaction mixture, containing 0.5 ml of 1.0% starch, 0.3 ml of 0.1 M of sodium acetate buffer (pH 5.5) and 0.1 ml of water were taken in the test tube and incubated at 55℃ for 10 min. for equilibration. The reaction was started by the addition of 0.1 ml of enzyme solution and allowed to proceed for 5 min. The reaction was stopped by the addition of 0.5 ml of 1 M HCl and chilled rapidly to room temperature. 0.2 ml volume of this reaction mixture was diluted to 15 ml by water containing 0.1 ml of 1 M HCl and 0.1 ml of iodine reagent (0.2% iodine in 2.0% KI). A Blank was prepared by adding 0.1 ml enzyme after stopping reaction by HCl. The absorbance was measured at 610 nm. An enzyme unit was defined as amount of enzyme, which caused a decrease in absorbance by 0.05 in starch iodine colour under assay conditions (b) DNS Method: The activity was measured according to the method of Bernfeld (1955) except that 0.1 M sodium acetate buffer, pH 5.4, was used. An enzyme unit was defined as the amount of enzyme required to produce 1 μmol of reducing sugar/min.

Protein assay

Protein was estimated by the method of Lowry *et al.*(1951) with crystalline bovine serum albumin as the standard. Specific activity was expressed as enzyme units/mg protein.

Enzyme purification: α-Amylase has been purified from 60 h germinated soybean (*Glycine max*) seeds by a succession of steps, which were carried out at 0 - 4℃, unless stated otherwise.

Isolation of α-amylase

Extraction

Germinated soybean seeds (60 g) were homogenized in a kitchen blender with 120 ml of extraction buffer (0.02 M Tris-HCl, pH 6.5). The homogenate was filtered through two layers of cheesecloth and centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge at 26,890 g for 15 min at 4℃. The clear supernatant was collected after filtration through the glass wool to remove any floating fat material.

Ammonium sulfate fractionation

Solid ammonium sulfate was added with stirring to 30% saturation. The pH was maintained at 6.5. The resultant suspension was stirred for 2 h and the precipitate was removed by centrifugation at 26,890 g for 20 min at 4℃. Supernatant was brought to 45% saturation with solid ammonium sulfate. After 2 h of stirring, suspension was centrifuged at 26,890 g for

Table 1. Purification of α-amylase from soybean seeds

Steps	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification (- fold)	Recovery (%)
Crude extract	6292	4303	1.31	1.00	100
Ammonium sulfate fraction (30-45% saturation)	434	240.4	1.81	1.38	89.7
Heat treatment	287	8.05	5.09	3.89	84.7
Sephadex G-75	23.67	1.125	21.04	16.33	48.9

20 min at 4℃, the supernatant was rejected and the precipitate was collected by centrifugation and suspended in a small volume of extraction buffer (5 ml).

Heat treatment

The enzyme from ammonium sulfate fractionation was subjected to heat treatment. It was kept at 70± 1℃ in an LKB water bath with constant shaking. After 10 min, the enzyme solution was chilled rapidly. The precipitated material was removed by centrifuged at 26,890 g, for 20 min at 4℃ and the clear supernatant collected.

Gel-filtration on Sephadex G-75

The above solution was passed through a Sephadex G-75 column (1.8 × 31 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.5, containing 3 mM $CaCl_2$ and 0.1 M NaCl. The column was successively washed with the same buffer to elute the enzyme from the column. The eluate was collected in 1 ml fractions at a flow rate of 20 ml/hr. The A_{280} was monitored of all fractions and the activity and protein of the peak fractions assayed. The high specific activity fractions were pooled and concentrated. The purified enzyme was stored at 4℃.

NATIVE-PAGE

Native-PAGE was performed according to the method of Laemmli (1970) using 10% acrylamide gel. The gel was stained for protein with Coomassie Brilliant Blue R250.

Effect of pH

The effect of change in pH on -amylase was studied. Starch solutions of different pH ranging from 4.0 to 10.6 in different buffers *viz.*, acetate buffer (pH 4.0-5.6), phosphate buffer (pH 5.7-8.0), Tris buffer (pH 7.2-9.0), glycine-NaOH (pH 8.6-10.6) was used to see the effect of pH.

Effect of temperature

The effect of temperature on α-amylase was studied by keeping the enzyme at different temperature ranging from 25-75℃ for 10 min in a water bath (Multitemp Bath, LKB, Sweden).

K_m and V_{max} studies

The enzyme obtained after gel-filtration chromatography was used for these studies. Rates were determined at various concentrations of substrate concentrations (0.5 to 10 mg/ml). The K_m and V_{max} were calculated from Lineweaver-Burk plot.

Substrate specificity

Substrates taken for substrate specificity were amylopectin, maltose, and starch. Assay was performed by DNS method, as amylopectin, maltose cannot form complex with iodine. Therefore, on the basis of amount of sugar released, one can determine the enzyme activity.

Inhibition studies

For inhibition studies, concentrations of inhibitors chosen were *viz.*, mercuric chloride (1 μM, 5 μM), copper chloride (0.1 mM, 0.5 mM) and zinc acetate (0.1 mM, 0.5 mM).

RESULTS AND DISCUSSION

Purification of α-amylase

α-Amylase activity is low in ungerminated seeds. It increases on germination, achieves a maximum value at 60 h of germination, and then declines if the seeds are allowed to germinate for longer periods (Fig. 1).

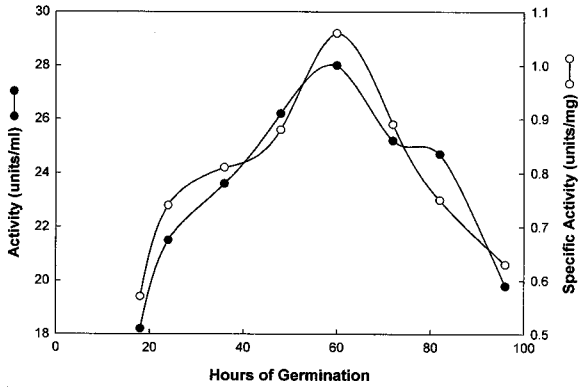

Fig. 1. Tripathi, Dwevedi and Kayastha.
Effect of germination on α-amylase activity and specific activity.

α-Amylase has been purified to approximately 16 fold to an apparent electrophoretic homogeneity. A sample protocol of the purification of α-amylase from 60 h germinated seeds is given in Table 1. The specific activity of final preparation was 21 units/mg protein and the recovery was about 50%, which varied slightly from batch to batch. The first two steps (ammonium sulfate fractionation and heat treatment) do not bring about any significant increase in specific activity but help by making the subsequent step more effective. It may be further noted that though the crude extract is not stable at 70℃, the presence of ammonium sulfate stabilized the enzyme against heat denaturation at this temperature. The heat-treated enzyme was passed through a Sephadex G-75 column, the elution profile showed two peaks (Fig. 2). Only peak I had the enzyme activity, indicating that heat treatment has denatured all high molecular weight proteins. Since amylases are known to have high tyrosine and tryptophan content, therefore detection at A_{280nm} during filtration was helpful. The purified enzyme gives a single band following PAGE in the absence of SDS (Fig. 3). Thus the final preparation obtained after gel-filtration is free from other impurities. The purified enzyme stored at 4℃ in extraction buffer was stable for more than 3 months without appreciable loss in activity.

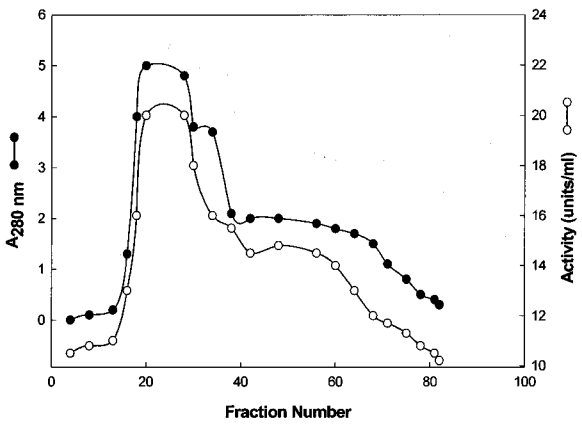

Fig. 2. Tripathi, Dwevedi and Kayastha.
Elution profile of α-amylase on Sephadex G-75 chromatography; (○) Enzyme activity, (●) A_{280nm}. The enzyme was eluted from the column with 20 mM Tris-HCl buffer containing 3 mM $CaCl_2$ and 0.1 M NaCl.

Substrate specificity

Some compounds were tested as substrates and the results are shown in Table 2. Compared to starch, amylopectin and maltose were hydrolyzed by α-amylase to only 24 and 5%, respectively. Thus, the data shows that high molecular weight substrates

like, starch is a preferred substrate for α-amylase from soybean. However, in case of pea leaf endoamylase, amylose followed by amylopectin and starch were the best substrates (Ziegler, 1988).

Effect of pH

The pH dependence of the purified α-amylase is shown in Fig. 4. Optimum activity of α-amylase was found to be at pH 7.6. α-amylases from different sources were shown to have pH optima in the range of 4.5-6.0 (Maeda *et al.*, 1978; Masuda *et al.*, 1987) and 5.5-6.5 (Beers and Duke, 1990). However, α-amylase of potato tubers was reported to have optimum pH in the range of 7.2 to 8.0 (Wolfgang and Sauter, 1996). Optimum pH of *Vigna mungo* α-amylase was reported to be 6.0 (Koshiba and Minamikawa, 1981).

Effect of temperature

Optimum temperature of α-amylase from soybean was found to be 55℃. It showed a Q_{10} value of 1.85 and energy of activation (E_a) was determined to be 12 kcal/mol. Amylase from cotyledons of germinating *Vigna mungo* seeds showed E_a to be 10.8 kcal/mol (Koshiba and Minamikawa, 1981). Temperature optima of α-amylases from different sources were reported to vary between 35-50℃ (Greenwood *et al.*, 1965; Bhalla and Altosar, 1984; Masuda *et al.*, 1987).

Table 2. Substrate specificity of α-amylase

Substrates	Activity (units/ml)	Activity relative to starch (%)
Starch	50.80	100.00
Amylopectin	12.18	24.26
Maltose	2.50	4.92

Fig. 3. Tripathi, Dwevedi and Kayastha.
8% Native-PAGE of α-amylase from soybean (40g). Protein band was visualized by staining with Coomassie Brilliant R250 Blue.

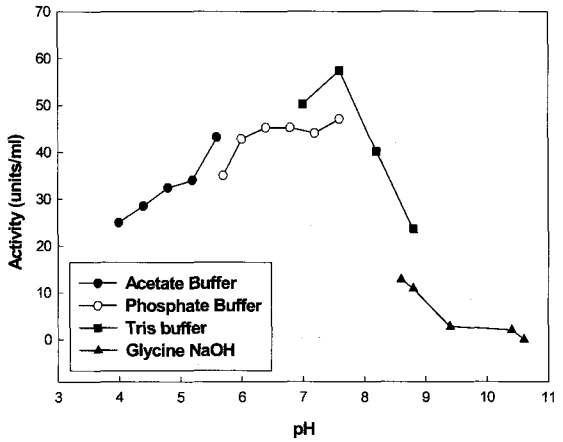

Fig. 4. Tripathi, Dwevedi and Kayastha.
Effect of pH on the activity of soybean α-amylase. The activity was checked in different buffer systems ranging from 4.0 to 10.6 *viz.*, acetate buffer (pH 4.0-5.6), phosphate buffer (pH 5.7-8.0), Tris buffer (pH 7.2-9.0) and glycine-NaOH (pH 8.6-10.6).

Table 3. Effect of various additives (5 mM each) on α-amylase activity

Additives	Units/ml* (a)	Units/ml* (b)
Control	48.2	45.4
DTT	47.6	44.8
PMSF	48.4	50.2
EDTA	48.1	39.7

* (a) and (b) are activities on first and tenth day.

Table 4. Effect of inhibitors on α-amylase activity

Inhibitors	Activity (units/ml)	Inhibition (%)
Mercuric chloride	38	21.32
Zinc acetate	43	10.97
Copper chloride	42.8	11.39

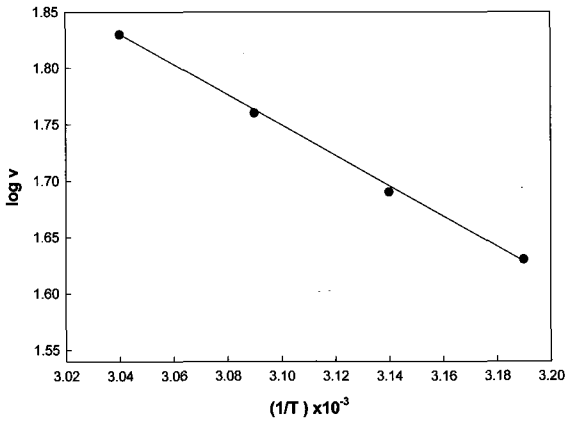

Fig. 5. Tripathi, Dwevedi and Kayastha.
Arrhenius plot (log v *versus* 1/T) for the effect of temperature on α-amylase activity.

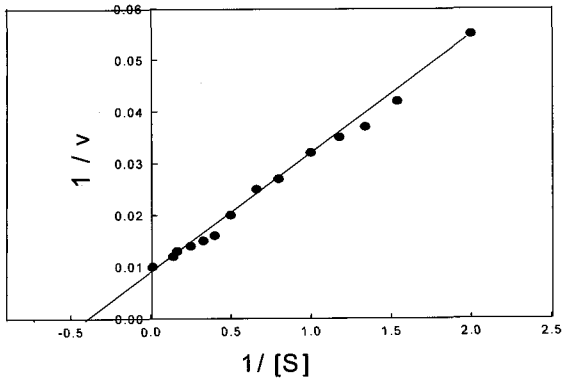

Fig. 6. Tripathi, Dwevedi and Kayastha.
Effect of substrate concentration on α-amylase activity shown as double reciprocal plot (1/v versus 1/[S]). Assay conditions have been described in the text.

Effect of additives

Results of various additives on the activity of α-amylase from soybean are shown in the Table 3 as tested on first day (a) and tenth day (b). From the data is evident that EDTA reduces the activity of the amylase showing amylase requiring calcium ion for its activity is chelated by EDTA. Incubation of the α-amylase with 5 mM EDTA for 20 min. resulted in about 50% loss of activity in *V. mungo* (Koshiba and Minamikawa, 1981). Furthermore, use of PMSF enhanced the activity showing the fact that proteases, if any present, were inhibited. Finally, DTT did not show any effect, indicating that cysteine is probably not involved in the activity of the enzyme.

Calculation of K_m and V_{max}

The apparent K_m and V_{max} values were determined for soluble starch at pH 5.4. The K_m and V_{max} were found to be 2.63 mg/ml and 52.63 mg/ml/min,

respectively from germinating soybean seeds (Fig. 5). K_m of α-amylase using soluble starch was reported to be 0.2 mg/ml from pea (Swain and Dekker, 1966), 16 mg/ml from sugar beet (Masuda *et al.*, 1987), 0.8 mg/ml from cotyledons of *Vigna unguiculata* (Bastos *et al.*, 1994).

Inhibition studies

The results of the inhibition studies were carried out with 0.5 mM of zinc acetate, copper chloride and 0.5 μM of mercuric chloride concentrations are shown in Table 4. Control had the activity of 48.3 units/ml. From the table, it can be concluded that the heavy metal ions are inhibitors of α-amylase from soybean seeds. In case of pea leaf endoamylase, mercury proved to be extremely potent, was nearly 100-fold more effective than copper. Zinc was strongly inhibitory only at millimolar concentrations, and 10 mM nickel was required for 50% inhibition (Ziegler, 1988). Plant amylases from different origin and under different physiological conditions have been reported to be inhibited by Zn^{2+} (Irshad *et al.*, 1981).

ACKNOWLEDGEMENTS

Financial assistance of the Council of Scientific and Industrial Research, New Delhi (Junior Research Fellowship to P.T.) is gratefully acknowledged.

REFERENCES

Bastos JLP, Prisco JT, Gomes FE.(1994) Purification and characterization of cotyledonary α-amylase from cowpea seedlings. *Revista Brasilera de Fisiologia Vegetal.* **6,** 33-39.

Beers EP, Duke SH. (1990) Characterization of α -amylase from shoots and cotyledons of pea (*Pisum sativum* L.) seedlings. *Plant Physiol.* **92,** 1154-1163.

Bernfeld P. (1955) Enzymes of carbohydrate metabolism: Amylases, α and β. *Methods in Enzymol.* **1,** 149-158.

Bewley JD, Black M. (1978) *Physiology and Biochemistry of Seeds.* pp. 245-269. Springer-Verlag, Berlin-Heidelberg-New York.

Bhalla RS, Altosar I. (1984) Purification and some properties of the extracellular α-amylase from *Aspergillus awamori. Can. J. Microbiol.* **31,** 149-153.

Chatterton RT, Vogelsong KM, Lu Y, Hudgens GA. (1997) Hormonal responses to psychological stress in men preparing for skydiving. *J. Clin. Endocrinol.* **82,** 2503-2509.

Fuwa H. (1954) A new method of microdetermination of amylase activity by the use of amylose as the substrate. *J. Biochem. (Tokyo)* **41,** 583-603.

Greenwood CT, MacGregor AW, Milne EA. (1965) The α-amylase from broad beans: Purification and properties. *Arch. Biochem. Biophys.* **112,** 459-465.

Irshad M, Goel M, Sharma CB. (1981) Effect of Zn^{2+} on plant α-amylases *in vitro. Phytochemistry* **20,** 2123-2126.

Koshiba T, Minamikawa T. (1981) Purification by affinity chromatography of α-amylase a major amylase in cotyledons of germinating *Vigna mungo* seeds. *Plant Cell Physiol.* **22,** 979-987.

Hemmingsen SH. (1980) Applied Biochemistry and Bioengineering, Vol. **2,** *Enzyme Technology* (Wingard, L.B. Jr., Katchalski-Katzir, E. and Goldstein, L.A. eds), pp. 157-183, Academic Press.

Jensen VJ, Rugh S. (1987) Industrial scale production and application of immobilized glucose isomerase. *Methods Enzymol.* **136,** 356-370.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Laemmli UK. (1970) Cleavage of the structure proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

Maeda I, Kiribuchi S, Nakamura M. (1978) Digestion of barley starch granules by the combined action of α- and β- amylases purified from barley and barley malt. *Agric. Biol. Chem.* **42,** 259-267.

Moselry MH, Keay L. (1970) Purification and characterization of the amylase of *Bacillus subtilis*NRRL B3411. *Biotechnol. Bioeng.* **12,** 251-271.

Masuda H, Takahashi T, Sugawara S. (1987) Purification and properties of starch hydrolyzing enzymes in mature roots of sugar beets. *Plant Physiol.* **84,** 361-365.

Robert X, Gottschalk TE, Haser R, Svensson B, Aghajari N. (2002) Expression, purification and preliminary crystallographic studies of α-amylase isozyme 1 from barley seeds. *Acta Cryst.* **D58,** 683-686.

Swain RR, Dekker EE. (1966) Seed germination studies.1. Purification and properties of α-amylase from cotyledons of germinating peas. *Biochim. Biophys. Acta* **122,** 75-86.

Walsh NP, Blannin AK, Clark AM, Cook L, Robson PJ, Gleeson M. (1999) The effects of high intensity intermittent exercise on saliva IgA, total protein and α-amylase. *J. Sports Sci.* **17,** 129-134.

Wolfgang W, Sauter JJ. (1996) Purification and properties of a starch granule degrading α-amylase from potato tuber. *J. Exp. Bot.* **47,** 1789-1795.

Yamaguchi M, Kanemaru M, Kanemori T, Mizuno Y. (2003) Flow-injection-type biosensor system for salivary amylase activity. *Biosens. Bioelectron.* **10,** 835-840.

Zeigler P. (1988) Partial purification and characterization of the major endoamylase of mature pea leaves. *Plant Physiol.* **86,** 659-666.