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Effect of Metal Ions, EDTA and Sulfhydryl Reagents on Soybean Amylase Activity

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ABSTRACT

Effect of some metal ions, EDTA and sulfhydryl reagents on the activity of partially purified amylase (Sp. Activity 1213 U mg^{-1} protein) of soybean seeds were studied. Cobalt (II) and Manganese (II) exhibited marked activating effects on the activity, enhancing up to 200% of the initial activity at 2 mM concentration while Mercury (II) ions severely inhibited. Inhibition by mercury and activation by cobalt and manganese were concentration-dependent. However, other metal ions (K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+}, Zn^{2+} and Fe^{3+}) moderately increased the enzyme activity to a certain extent and then suppressed. Sodium, Cadmium and Nickel had no detectable influence on the activity. EDTA (12.5 mM) was found to be ineffective even for 1 h of incubation at 27°C suggesting that no metal ion is present in the enzyme. No marked inhibition of amylase activity with the sulfhydryl reagents was found. The stability of the enzyme to metal ions as well as EDTA suggests its promising potential for use in detergent industries.

Key words: Amylase, partial purification, metal ions, chelating reagents, detergent formulations

INTRODUCTION

Amylases are among the most important enzymes used for several biotechnological applications particularly employed in starch processing industries for the hydrolysis of polysaccharides such as starch into simpler sugar constituents (Haq *et al*., 2002) and this is the basis for various industrial processes like preparation of glucose syrups, bread making, brewing, etc. These enzymes account for about 30% of the world's enzyme production (Van der Maarel *et al*., 2002). Besides, it also finds use in detergents, desizing of textiles, modified starches, hydrolysis of oil-field drilling fluids and paper recycling (Richardson *et al*., 2002).

Many substances alter the activity of an enzyme by combining it in a way that influences the binding of the substrate (Dogan *et al*., 2007). These substances can be called as effectors either being activators or inhibitors. Metal ions can be considered as good examples, different metals exhibiting different behaviors in their ability to act as effectors. Li *et al*. (2005) also stated that metal binding to enzymes plays an important role in their activation and stabilization. Most amylases are known to be metal ion dependent enzymes (Pandey *et al*., 2000; Gupta *et al*., 2003; Ramachandran *et al*., 2004). According to Leveque *et al*. (2000), a metal ion present at high concentrations might compete with another metal present at a lower concentration and replace it at a metal-binding site, even if its affinity for the binding site is lower.

An accepted property of amylases is their content of Ca^{2+} as an integral part of the enzyme molecule and their consequent activation by Ca^{2+} and inhibition by chelating reagents

(Mar *et al*., 2003; Kiran and Chandra, 2008). Besides, amylases have been reported to be inhibited by heavy metal ions such as Ag^{+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, etc., suggesting the involvement of cysteine residue in enzyme activity. The importance of cysteine residues for catalysis has been described for amylases independent of their origin (Pandey *et al*., 2000; Lo *et al*., 2001; Díaz *et al*., 2003). The thiol of a cysteine residue occasionally acts as a ligand of metal ions (Tatara *et al*., 2005). Amylases are also found sensitive to various sulfhydryl modifying reagents as free thiol or sulfhydryl groups are considered to be useful in stability, redox behaviour, metal binding, acidity, nucleophilicity and catalytic activity (Bardwell, 2005; Giles *et al*., 2003).

In order to obtain an insight into these, an attempt has been made to partially purify amylase of soybean seeds and the effect of various metal ions and the chelating reagent; EDTA on the amylase activity was determined. Besides, the possible effects of sulfhydryl reagents (p-hydroxymercurybnzoic acid, N-ethylmaleimide and iodoacetic acid) on amylase activity have also been investigated.

MATERIALS AND METHODS

Materials: Soybean seeds were procured from the local market. Sephadex G-75 was from Pharmacia Fine Chemicals Uppsala, Sweden. Bovine serum albumin and maltose were obtained from Sigma Chemical Co., USA. Soluble starch and ammonium sulphate were from Qualigens Fine Chemicals, Mumbai. All other reagents were analytical grade chemicals either from BDH or E. Merck, India. The study was conducted during January 2009 to December 2009.

Enzyme extraction and preparation of crude extract: Soybean seeds (50 g) were soaked in 25 mM sodium acetate buffer (pH 5.5) for 8 h at 4°C. The soaked seeds were homogenized along with the buffer using a kitchen blender, filtered through muslin cloth and centrifuged at 15,000 rpm (21,633 g) for 30 min. The supernatant so obtained (crude preparation) was subjected to further purification.

Enzyme and protein assays: The amylase activity was determined by the method of Bernfeld (1955) with slight modifications. The assay system contained 1.0 mL of suitably diluted enzyme solution, 0.5 mL of 2% soluble starch (in assay buffer) and 0.5 mL of assay buffer (Tris acetate, 150 mM, pH 5.5). This reaction mixture was incubated at 27°C for 3 min. The reaction was terminated by the addition of 2.0 mL of DNS (3,5-dinitrosalicylic acid) reagent solution. Colour, due to the reducing sugar liberated was developed by heating the reactants in a boiling water bath for 5 min and then cooling down to room temperature. After addition of 20 mL of double distilled water, the absorbance was determined at 540 nm. A blank was prepared as above but without the enzyme. A calibration curve of maltose was also prepared.

One unit of amylase activity was defined as the amount of enzyme required to produce 1 μmol of maltose per minute under the assay conditions.

The alkaline 3,5-dinitrosalicylic acid reagent occasionally gives anomalous results, due to the effect of various ions on the reducing value (Robyt and Whelan, 1965).

Protein was estimated by the method of Lowry *et al*. (1951) with Folin-ciocalteau reagent calibrated with crystalline bovine serum albumin.

Enzyme purification

Ammonium sulphate precipitation: The crude amylase preparation (50 mL) was precipitated with solid ammonium sulphate. The sample was separated into three fractions based on the

saturation percent of ammonium sulphate (0-30, 30-80 and 80-100%). The precipitation was carried out at 4°C under constant stirring and the precipitated proteins were centrifuged at 10,000 rpm (9615 g) for 15 min. The precipitate obtained was dissolved in the extraction buffer (25 mM sodium acetate, pH 5.5). Specific activity of the sample was estimated in all the three fractions. The selected fraction having high specific activity was further processed by dialysis (against the same extraction buffer) and centrifugation (9615 g, 15 min) prior to gel filtration chromatography.

Sephadex G-75 gel filtration chromatography: The enzyme obtained from the previous step was applied (1.0 mL) to a Sephadex G-75 column (1.4×32 cm) pre-equilibrated with 25 mM sodium acetate buffer (pH 5.5) and eluted with the same buffer. The eluate collected in various 2 mL fractions were analyzed for both protein and amylase activity. The active fractions were pooled and concentrated against solid sucrose. The enzyme preparation was kept frozen (at-20°C) in small aliquots until further use.

Electrophoresis: 12% Native polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). The bands were visualized by coomassie blue and silver staining in order to check the purity of the enzyme preparation.

Effect of metal ions, EDTA and sulfhydryl reagents on amylase activity: A stock solution of each of the desired reagent was prepared in double distilled water. The residual amylase activity of suitably diluted enzyme was determined in the presence of varying concentration of these reagents added in the standard assay mixture (comprising of 2% starch, 150 mM Tris acetate buffer, pH 5.5 at 27°C). In order to explore the stability of the enzyme to the chelating reagent EDTA, enzyme was incubated with various concentrations of EDTA (direct exposure) for 1 h at 27°C at pH 5.5 and then the residual activity of the exposed enzyme was determined.

RESULTS AND DISCUSSION

Partial purification of the enzyme: Among the three fractions selected for the study, protein precipitated in the range of 30-80% saturation of ammonium sulphate (having a specific activity of 300 U mg^{-1} protein) was selected for further separation on Sephadex G-75 column. Taking into account the amylase obtained in a single symmetrical peak from fractions 8-22, 5.8 fold purification was achieved with recovery of 51% (Table 1 and Fig. 1). Recently, Mohamed *et al.* (2009) partially purified amylase from a wheat local variety with 1.41 fold purification and 2.3% recovery.

Effect of metal ions on soybean amylase activity: Various metal ions such as K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Na^+, Cd^{2+}, Ni^{2+}, Hg^{2+}, Pb^{2+}, CO^{2+}, Mn^{2+} at various concentrations

Table 1: Partial purification of α-amylase from soybean seeds

Sample	Volume (mL)	Total activity U	Total protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude enzyme	50	66065	316	209	100	1
Ammonium sulphate precipitation	43	61858	206.4	300	94	1.4
Sephadex G-75 gel filtration	42	33633	50.4	1213	51	5.8

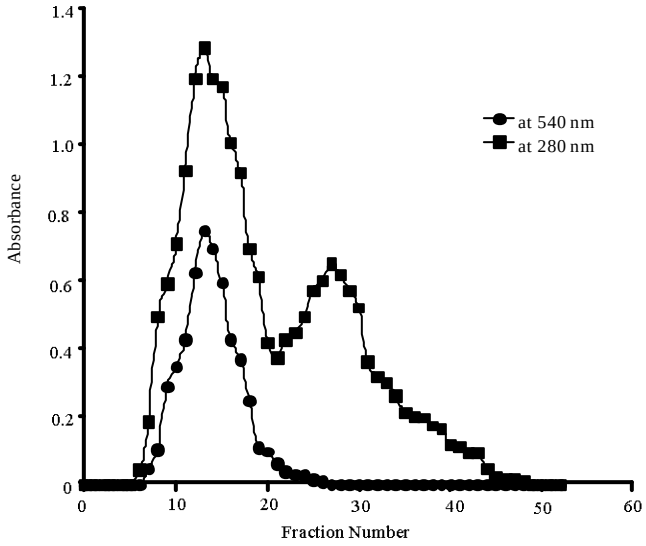

Fig. 1: Purification profile of soybean amylase in Sephadex G-75 gel filtration chromatography

(ranging from 0.0005 to 5 mM) were tested for activation/inhibition effects. Na^{+}, Cd^{2+} and Ni^{2+} had negligible effect on amylase activity (data not shown). K^{+}, Mg^{2+}, Al^{3+} and Fe^{3+} showed an increase in amylase activity upto 0.005 mM and then showed a gradual decline with increasing concentrations (Fig. 2). Similarly, the activating effect of Ca^{2+}, Cu^{2+} and Zn^{2+} on amylase was dependent on the concentration of the metals upto the limiting value of 0.25 mM, beyond which there was a decrease in activity. Usually, the role of Ca^{2+} in maintaining the stability and structure of the amylase is well documented (Parkin, 1993). Ca^{2+} was found to increase the activity of α-amylase from *Bacillus subtilis* (El-Banna *et al*., 2007). Cu^{2+} showed strong inhibition on the activity of the enzyme from *Bacillus subtilis* JS-2004 (Asgher *et al*., 2007). The effect of Zn^{2+} varied between amylases. For instance, it had a potent inhibitory effect on *Schwanniomyces alluvius* α-amylase (Moranelli *et al*., 1987).

A concentration-dependent and time-independent inhibition in enzyme activity was observed in case of heavy metal ion Hg^{2+} (observed from 0.001 to 5 mM, Fig. 3). A similar trend with respect to concentration-dependence was observed in case of Pb^{2+} which showed complete loss in activity at 50 mM (data not shown). Hg^{2+}, Pb^{2+} and Cu^{2+} have been reported to inhibit microbial amylase (Lin *et al*., 1998). Hg^{2+} was observed to be a potent inhibitor in α-amylase from mung beans (Tripathi *et al*., 2007). The inhibitory effect of Hg^{2+} on amylase may indicate the importance of indole amino acid residues in enzyme function as has been demonstrated for other microbial amylases (Gupta *et al*., 2003).

A significant stimulation in amylase activity was observed with increasing concentration of CO^{2+} and Mn^{2+} (observed from .0005 to 2 mM, Fig. 4) which are of wide occurrence in biological systems. Dahot *et al*. (2001) also found an increase in amylase activity with CO^{2+} and Mn^{2+} with *Moringa oleifera* seeds. Similarly, Dutta *et al*. (2006) obtained an enhanced amylase activity in *Heliodiaptomus viduus* with these ions (at 5 mM). In contrast, an inhibitory effect of CO^{2+} on amylase from *B. subtilis* JS-2004 was observed (Asgher *et al*., 2007). *Penicillium olsonii* α-amylase showed an enhanced enzyme activity with Mn^{2+} (1 mM) while no effects in enzyme activity was found with CO^{2+} (Afifi *et al*., 2008).

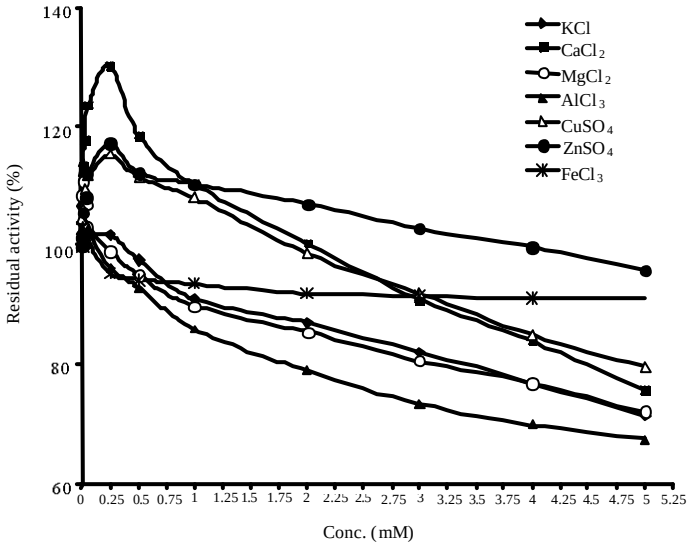

Fig. 2: Effect of metal ions on the activity of amylase of soybean seeds

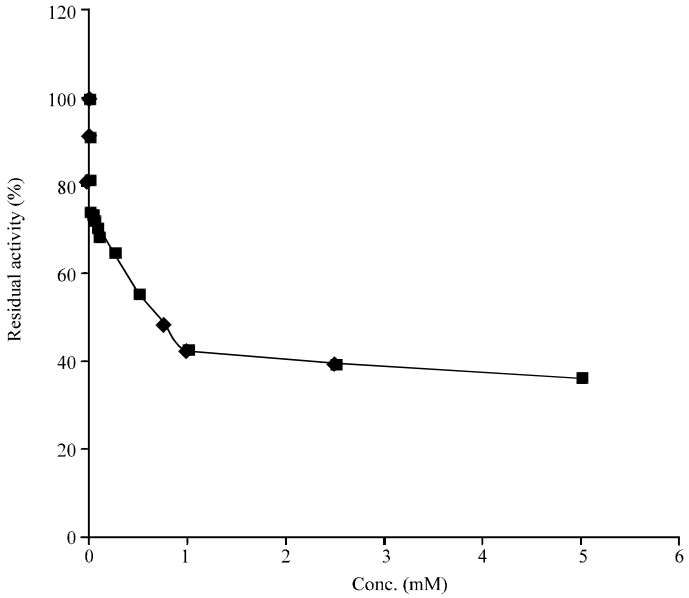

Fig. 3: Effect of the heavy metal ion Hg^{2+} on soybean amylase activity

Reports have been made that metallic chlorides are potential activators of amylases (Vega-Villasante *et al*., 1993; Oboh and Ajele, 1997; Mohapatra *et al*., 1998). If this is the case, then the metallic chlorides used (viz. KCl, $CaCl_2$, $MgCl_2$ and $FeCl_3$) should show activation virtually at all the concentrations tested. Moreover, if it were responsible alone, one would expect the degree of activation to be the same for all the metallic chlorides tested. However, results as already described are contrary to this. This argument is in line with the findings of Wakim *et al*. (1969) which showed that halides activate amylase activity but they are not mandatory for the activity of the enzyme. Florkin and Mason (1962) also stated that amylases are either activated by this anion or are indifferent to its presence. Thus, it can be said that the effects observed on the enzyme activity might be the contribution from cations.

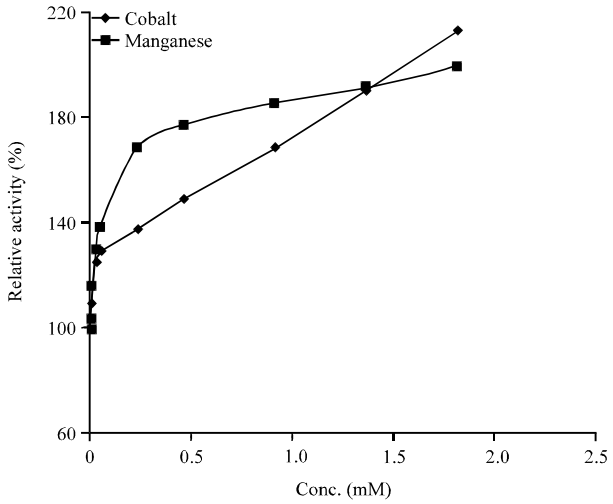

Fig. 4: Effect of cobalt and manganese on soybean amylase activity

Effect of EDTA on soybean amylase activity: It is well known that amylases contain Ca^{2+} as an essential component of the enzyme molecule and are often inhibited by the chelating reagent EDTA (Mar *et al.*, 2003; Kiran and Chandra, 2008) presumably because of its chelating properties. Interestingly, soybean amylase retained almost full activity with the varying concentrations of EDTA (from 0.0005 to 12.5 mM) used in the study. Besides, the enzyme maintained about 90% of its initial activity even after 1 h incubation with 12.5 mM EDTA (Fig. 5). Utong *et al.* (2006) also found continued production of extracellular amylase activity suggesting that the amylase enzyme produced was not a metalloenzyme. In contrast, Femi-Ola and Olowe (2011) found that EDTA inhibited the enzyme activity suggesting that EDTA acted by chelating Ca^{2+}. These results indicate that soybean amylase is strongly resistant to the chelating reagent which is very interesting in that no metal ion is essential for the manifestation of soybean amylase activity. Moreover, its stability to incubation with chelating reagents (one of the indispensable ingredients in detergent formulations) is of great importance from the viewpoint of its high performance even in detergent formulations and the physiological significance of which calls for study in future works.

Effect of sulfhydryl reagents on soybean amylase activity: The effect of the sulfhydryl reagents (p-hydroxymercurybenzoic acid, N-ethylmaleimide and Iodoacetic acid; observed from 0.5 to 12.5 mM) on amylase activity are presented in Fig. 6. The minimum amount of these reagents inducing a detectable inhibition of activity in soybean amylase was found to be 1 mM. Iodoacetic acid and N-ethylmaleimide showed decline in enzyme activity with increasing concentrations while pHMB had only marginal inhibitory effects on the activity of soybean amylase. This pattern of sulfhydryl reagent selectivity is not like that reported for other plant amylases which are largely inhibited by low concentrations of any sulfhydryl reagent (Marshall, 1975). Besides, all sulfhydryl reagents used in this study should have totally inhibited activity if cysteinyl sulfhydryls were necessary for catalysis. However, the data indicate that soybean amylase sulfhydryls are not directly involved in catalysis. Soybean amylase inhibition by the sulfhydryl reagents could be due to binding of noncatalytic cysteinyl sulfhydryls, causing changes in the alignment of catalytic amino acids, as is proposed for sweet potato α-amylase (Thoma *et al.*, 1971) or by causing steric hindrance.

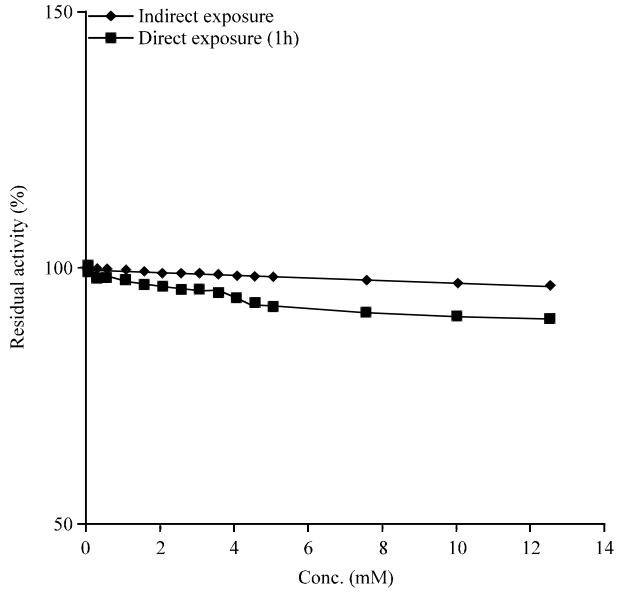

Fig. 5: Effect of indirect exposure (in the presence of substrate) and direct exposure of soybean amylase (1 h) to EDTA

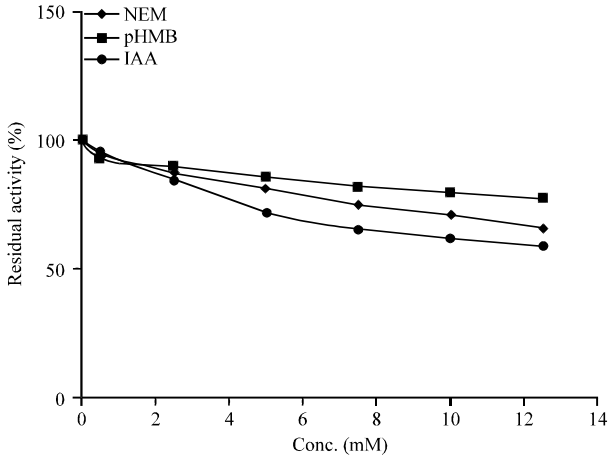

Fig. 6: Effect of sulfhydryl reagents on activity of soybean amylase

CONCLUSION

The soybean amylase was partially purified by ammonium sulphate fractionation and gel filtration chromatography with a purification factor of 5.8 and a recovery of 51%. The enzyme retained more than 65% activity even in the presence of metal ions at a concentration of 5 mM. It was also found quite stable to the effect of chelating reagent, EDTA. No significant inhibition with the sulfhydryl reagents was found indicating that sulfhydryls of soybean amylase are intact and not free to be directly involved in catalysis. The stability of the enzyme to the metal ions and chelating reagent establishes its potential for use in detergent formulations.

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