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Purification and Characterization of α-Amylase from Apple (*Malus pumila)*

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ABSTRACT

Malus pumila (apple) was homogenized in buffer for α-amylase extraction. The crude extract showed 3.09 U/mL enzyme activity that was subjected to ammonium sulfate precipitation and 4.76 U/mL of activity was obtained with 5.01 U/mg specific activity. It was applied to sephadex G-150 for gel filtration chromatography. It indicated the activity of 5.025 U/mL and specific activity 38.95 U/mL with 20-fold purification. SDS-PAGE of enzyme showed that unwanted proteins are removed and clear band of enzyme was appeared. Molecular weight was determined by sephadex G-150 column that is 51,180 Dalton. Amylase showed a pH optimum of 6.8, temperature 37°C, K_m value of $2.0x10^{-3}$ g/mL, λ_{max} 540nm and incubation time for enzyme assay was 10 minutes. Increase in enzyme concentration showed a linear increase in amylase activity.

Key Words: Amylase; Apple; *Malus pumila*; Purification; Characterization

INTRODUCTION

Microbial amylases are potentially useful in pharmaceutical and fine chemical industries (Augustin *et al*., 1981; Pandey *et al*., 2000). Several methods have been developed for cereal amylolytic activity estimation but there is a lack of information about the application of these methods for fruits. *Musa paradisiaca* (Banana) achieve 20% of starch contents, which is degraded during ripening of the fruit involving α and β-amylase (Bassinello *et al*., 2002). Amylases are among the most important enzymes and are of great significance in present day biotechnology especially in bread baking process. After the addition of enzyme, bread volume increases and retains its softness (Ammar *et al*., 2002). The spectrum of applications has widened many other fields such as clinical, medical and analytical practices as well as their wide spread applications in starch saccarification, textile, food, brewing and distilling industries (Aktinson & Movituna, 1991).

α-Amylase is monomeric, calcium binding glycoprotein. Its single polypeptide chain has 496 amino acid residues with four disulfide bridges. In fruits amylase activity is correlated with the fruit ripening and climacteric rise in respiration. During ripening period, starch contents of fruits are degraded in a complex process involving α and β-amylases as well as α (1-4 &. 1-6) glucosidases (Lajalo, 2001).

The present investigation was carried out to extract, purify and characterize α-amylase from *Malus pumila* (apple/Kalakolu).

MATERIALS AND METHODS

Preparation of homogenate. Soft thalamus of apple (100 g) was taken in 400 mL of 0.1 M phosphate buffer (pH 7) and homogenized for 10 min. It was centrifuged at 10,000 rpm for 15 min at 4°C and supernatant was separated from the sedimented cellular debris (Zia & Andaleeb, 2002).

Analytical. The activity of α-amylase at various steps was determined by DNS method as described by Bernfeld (1955) and protein contents by Biuret method as described by Gornall *et al*. (1949).

Purification of α-amylase. Solid ammonium sulfate was added to the crude extract, until 40% saturation. It was centrifuged at 10,000 rpm for 15 min at 4°C after an incubation of 4 h at 4°C. Supernatant of 40% saturation was adjusted upto 60% saturation and again centrifuged at pre-mentioned conditions. Then the sediments were dialyzed against buffer to desalt the enzyme for 5 hours at 4°C (Khoo *et al.,* 1994).

A column of sephadex G-150 was prepared by heating the slurry at 90°C for 5 h, which was poured into the column. The desalted sample (2 mL) was poured onto the column which was eluted with phosphate buffer (pH 7) in 24 fractions of 2 mL each (Zia, 2002).

Electrophoresis. SDS-PAGE was applied after sephadex G-150 chromatography to analyze the purity and homogeneity of the purified enzyme (Laemmli, 1970).

Characterization

Effect of pH. Optimum pH of enzyme was determined as α-amylase was assayed at different pH ranging from 1-10.

Effect of temperature. Optimum temperature α-amylase was determined at various temperatures ranging from 5-75°C (Collins *et al.,* 1993).

Effect of substrate and enzyme. Enzyme was assayed with the modification that the assay mixture contained varying amounts of starch from 0.1-1.3% and enzyme concentration from 0.1-1.0 mL for the determination of maximum reaction

Fig. 1. Analysis of α-amylase after ammonium sulfate precipitation

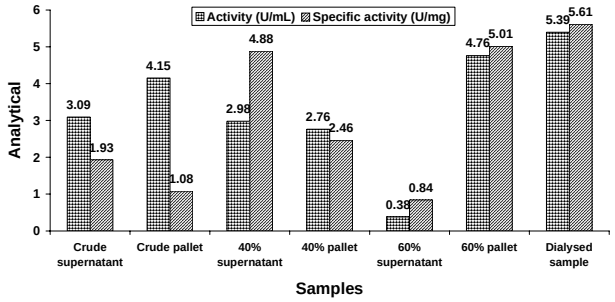


Fig. 2. Gel filtration analysis of α-amylase from apple

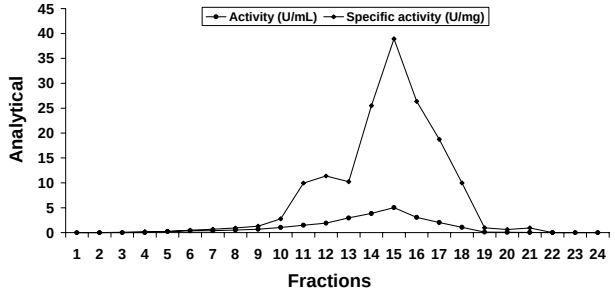


Fig. 3 Effect of pH on activity of α-amylase from apple

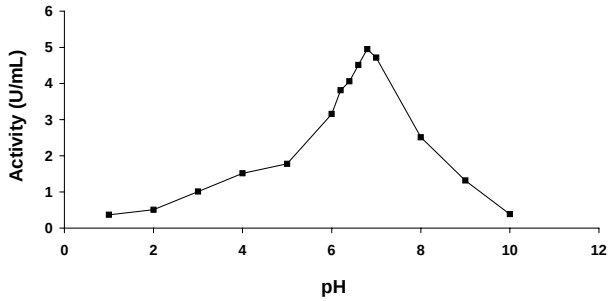
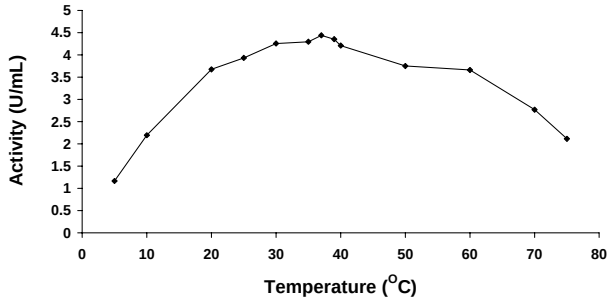


Fig. 4. Effect of temperature on α-amylase activity from apple



velocity (V_{max}) and Michaelis-Menten constant (K_m) (Khoo *et al.,* 1994).

Optimum wavelength. The assay mixture was carried out at different wavelength to obtain the optimum wavelength for the enzyme.

Molecular weight. It was determined by using sephadex G-150 column and bovine serum albumin, egg albumin, casein and cytochrome C were used as molecular weight markers. These were eluted with buffer and absorbance of fractions was noted at 280 nm and a graph was plotted.

RESULTS AND DISCUSSION

Kalakolu has been found to have the highest α-amylase activity. In this variety, unripe fruit has 0.25 U/mg of protein which increases approximately 8 folds with the ripening of the fruit (Patil & Magar, 1978). The crude extract having activity of 3.09 U/mL, was subjected to ammonium sulfate precipitation so three fold purification was achieved (Fig. 1). Ammonium sulfate is the most commonly used reagent for salting out the proteins because of its high solubility that permits the achievement of the solutions with higher ionic strength (Voet *et al*., 1999). Michelena and Castillo (1984) purified α-amylase from *Aspergillus foetidus* by $(NH_4)_2SO_4$ precipitation and achieved 11 fold purification while Nirmala and Muralikarishna (2003) obtained 26 fold of the enzyme from malted finger millet.

Using gel filtration that involves the separation of the molecules on the basis of molecular weight carried purification. It has been observed that maximum activity 5.025 U/mL obtained in the 15th fraction with 38.95 U/mg of specific activity and 20.18 fold purification (Fig. 2). Chang *et al*. (1995) employed the enzyme to the same conditions but the enzyme obtained 16 fold purification of from *Aspergillus oryzae*. According to Nirmala and Muralikarishna (2003), 31 folds of the said enzyme was achieved from finger millet. These differences are due to different sources of α-amylase and environmental features in this regard. Various fractions obtained during the study were subjected to SDS-PAGE to seek the homogeneity and purity of the enzyme.

Enzyme showed the optimum activity at pH 6.8 (Fig. 2) with activity of 4.95 U/mL and by increasing or decreasing the pH from the optimum pH the activity of enzyme was decreased. Michelena and Castillo (1984) also showed 6.6 optimum pH for the enzyme. Comparative study regarding thermal effects on alpha-amylase revealed that with increasing temperature from 0-37°C, activity of enzyme was maximum. With further increase in temperature, there was a drastic decrease in activity, which might be due to denaturation of enzyme. The optimum temperature was found to be 37°C indicating 4.75 U/mL activity (Fig. 4).

Fig. 5. Dependence of rate of α-amylase upon its concentration in reaction mixture

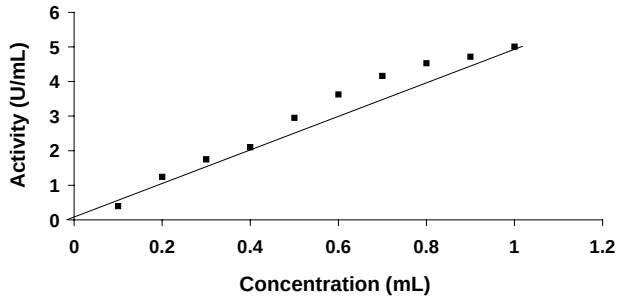


Fig. 6. Typical plot of initial velocity of α-amylase as a function of various concentrations of starch

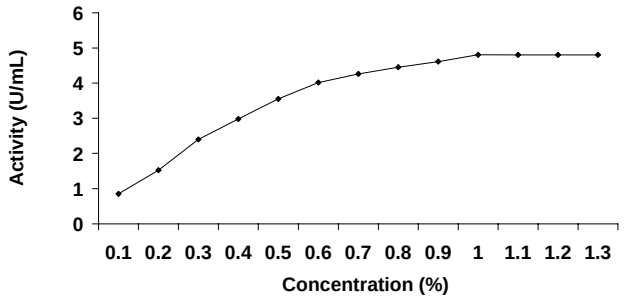


Fig. 7. Determination of Km for typical plot of initial velocities of α-amylase as a function of starch concentrations

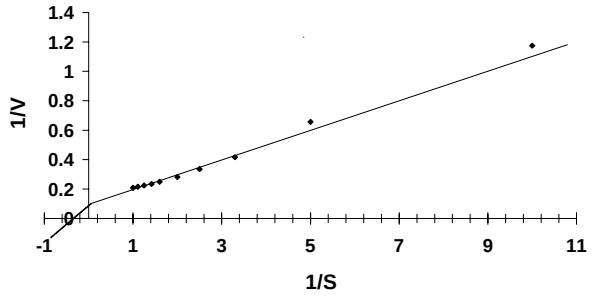


Fig. 8. Determination of incubation time for reaction mixture to estimate maximum α-amylase activity

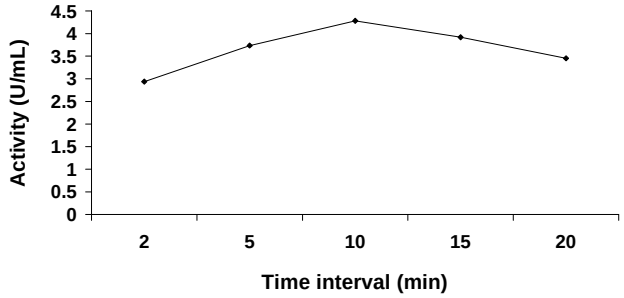


Fig. 9. Determination of wavelength that gives maximum activity for the reaction product mixture

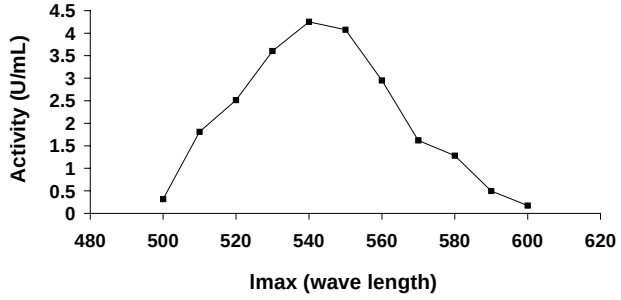
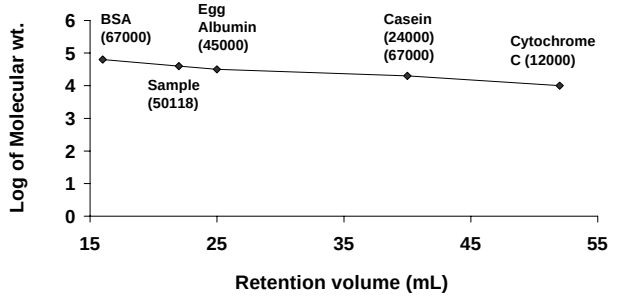


Fig. 10. Molecular weight determination for α-amylase from apple



Activity of enzyme was gradually and continuously increased with increasing the enzyme concentration as shown in Fig. 5. Activity of enzyme at 1.0% substrate concentration was 4.803 U/mL (Fig. 8) that proved to be optimum. The K_m value of enzyme obtained $2x10^{-3}$ g/mL (Fig. 7). The enzyme obtained maximum activity (4.283 U/mL) after the time interval of 10 min. (Fig. 8) in case of enzyme assay and it showed 540 nm as optimum wavelength (Fig. 9).

The molecular weight of enzyme was detected 51,180 Dalton. It was determined by using sephadex G-150 column. Bovine serum albumin (MW 67,000), egg albumin (MW 45,000), casein (MW 24,000) and cytochrome (MW 12000) were used as markers. These were eluted with buffer and absorbance of fractions was noted at 280 nm and a graph was plotted (Fig. 10). Yasin *et al*. (1981) characterized mango amylase with optimum pH of 5.5, temperature optimum 37°C, K_m $3.3x10^3$ g/mL and molecular weight of 53,000 Dalton. Annis (1982) reported that amylase from *Carica papaya* showed optimum pH 9 and temperature 37°C, K_m value of $3.3x10^3$ and molecular weight 52,000 Dalton.

CONCLUSION

It is concluded that apple is a rich source of α-amylase. So, it is recommended to exploit the natural sources for purification of α-amylase as well as other enzymes.

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