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EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF AMYLASE FROM APPLE (MALUS PUMILA)

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Amylases are among the most important enzymes and are of great significance in present day biotechnology and the spectrum of applications has widened many other fields such as clinical, medical and analytical practices. Malus pumila (apple) was homogenized in buffer for α-amylase extraction. The crude extract showed 0.2 U/mg specific activity that was when subjected to ammonium sulfate precipitation, 0.4 U/mg specific activity was obtained. After applied to sephadex G-100 for gel filtration chromatography, it indicated the specific activity of 4.76 U/mg with 24-fold purification. NATIVE-PAGE of enzyme showed that unwanted proteins are removed and clear band of enzyme was appeared. The enzyme was partially purified and had a high activity in a broad pH range of 6-8, with the maximal activity occurring at pH 6. The enzyme was stable between the temperatures of 30-50 ºC and the optimum temperature for maximal amylase activity was found to be 35 ºC, after which the enzyme activity dropped. In apple, amylase activity was increased by the addition of Ca^{2+} and Mn^{2+} and Mg^{2+} while Hg^{2+} showed inhibitory effect. The enzyme retained its 90% activity after incubation for 1 h at temperature of 40 ºC.

Keywords: Amylase; Apple; Malus pumila; Purification; Characterization

INTRODUCTION

Amylases are amongst the most studied enzymes (Noomen *et al.,* 2009). There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Priya *et al.,* 2011). Their enormous diversity of function makes them one of the most fascinating groups of enzymes for application at different sectors of life in both physiological and commercial fields (Marc *et al.,* 2002). Amylases are starch degrading enzymes that catalyze the hydrolysis of internal alpha 1-4 glycosidic bonds in polysaccharides with the retention of alpha anomeric configuration in the products (Takata *et al.,* 1992). They are found in all forms of organisms regardless of kingdom. The classification of starch digestive enzymes in malt as á and â -amylases according to the anomeric type of sugars produced by the enzyme reaction. Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyse from the non-reducing end, successively resulting in short end products. Today a large number of enzymes are known which hydrolyse starch molecule into different products and a combined action of various enzymes is required to hydrolyse starch completely.

Amylase have gained importance in various industrial process like pharmaceutical, food, brewing, paper, textile

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and chemicals. It is extensively used in pharmaceutical industries in digestive tonics, for hydrolysis of starch to produce different sugars like glucose and maltose which have several applications. Another industry which uses amylase is detergent industry. The use of detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Couto and Sanromán, 2006). The use of α-amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e., for the production of low-viscosity, high molecular weight starch (Gupta, 2003; vander Maarel *et al.*, 2002). Keeping in mind various application of amylase, the present investigation was selected with the aims of Isolation, characterization and partial purification of amylase from apple.

MATERIALS AND METHODS

Collection of Sample

Apples were procured from local market of Kurukshetra University Campus.

Extraction of Enzyme

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 sediment cellular debris.

Enzymatic Assay of Amylase

Quantitative estimation of enzymatic activity was done by enzymatic assay provided by Miller (1959). The protocol was standardized according to lab conditions. The enzyme assay used for amylase activity involved measuring the reducing sugars resulting from the enzymatic hydrolysis of soluble starch. A 0.15-ml enzyme extract was mixed with an equal volume of 2% soluble starch, mixed by swirling and incubated for exactly 3.0 minutes at 20 °C. Then added color reagent solution i.e., 0.1 M sodium potassium tartarate and 1ml of 96 mM 3,5-Dinitrosalicylic acid and the mixture was incubated at 30 °C for 10 min the mixture was Capped with a vented cap and place in a boiling water bath. Then added enzyme solution, boiled for 15 min and cooled at room temperature and took absorbance at 540 nm. The amount of reducing sugars produced was determined by the dinitrosalicylic acid method with maltose as the standard. One unit of amylase activity was defined as the amount of enzyme which liberated 1µmol of reducing sugars, with maltose as the standard, per min under the specified conditions (Miller, 1959).

Protein Estimation

Total protein contents of the enzyme solution were measured according to the method described by Lowry *et al.* (1951) using Bovine Serum Albumin (BSA).

Partial Purification

After extraction of enzyme it is further purified with the help of purification technique, i.e., ammonium fractionation, dialysis and gel filtration chromatography.

Ammonium Sulphate Fractionation

The crude enzyme was purified from the supernatant fluid using ammonium sulphate in a 0.1 M phosphate buffer of pH 6. For this purpose, various ammonium sulphate concentrations, i.e., 0-20, 20-30, 30-50 and 50-70% were used for the precipitation of enzymes. The respective levels were mixed in 200 ml of crude enzyme filtrate and kept at 4 °C for 1 to 2 hours with continuous and constant stirring. The precipitates were collected and analyzed for amylase activity.

Dialysis

Dialysis is basically carried out to remove traces of ammonium sulphate from enzyme extract because it may inhibit enzyme activity. Put the enzyme extract in dialysis bag which is than kept in a beaker containing distilled water of pH 6.5 and change distilled water after 4 hours and kept it in refrigerator for overnight. Enzyme is then removed from the dialysis bag and kept in test tube at 4 °C.

Gel Filtration Chromatography

The dialyzed enzyme fraction was further purified by gel filtration chromatography. It was loaded on sephadex G-100 column and eluted with Tris-HCl buffer (pH 7.4) with the flow rate of 0.5 ml/min. Total fractions (each 2 ml) were subsequently collected and its protein content was measured by using spectrophotometer at λ280. The fractions that have high absorbance at λ280 were collected and evaluated for its activity. The fractions showing higher enzyme activity were collected together for further characterization.

Characterization of Partially Purified Enzyme Effect of pH on Amylase Activity

The effect of pH on the activity of the amylases was determined by conducting the reaction at different pH values (0.5 M citrate-phosphate buffer, pH 3-7; Tris-HCl buffer, pH 7-8.5 and Glycine-HCl buffer, pH 9-11). The activity was measured using the standard assay conditions.

Effect of Temperature on Amylase Activity

Influence of temperature was determined by conducting the reaction at different temperature values (25-50 °C) for varying time period 10-60 minutes. The activity was measured using standard assay condition.

Effect of Metal Ions on Amylase Activity

The effects of various metal ions (20 mM) on enzyme activity were investigated by pre-incubating the enzyme preparation for 30 minutes at 35 °C in the presence of Na^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+} and Zn^{2+}) metal ions.

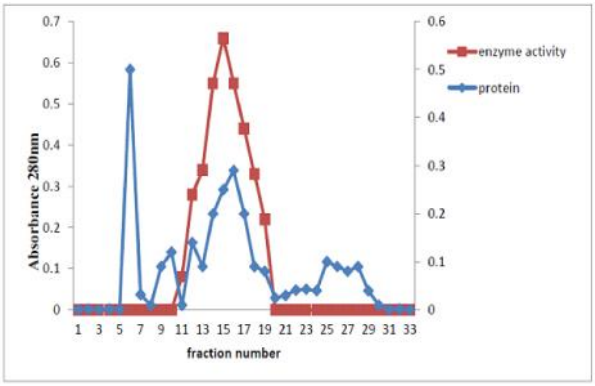
Effect of pH on Enzyme Stability

The effect of pH on amylase stability was studied by pre-incubating the enzyme in buffers of different pH values in the range of pH 4.0-11.0 for 1 h at 35 °C. The residual activities were determined at 35 °C, pH (6) and expressed as percentage of the initial activity taken as 100%.

RESULTS AND DISCUSSION

In the intensification of this topic the present work was done to isolate amylase from apple. The extraction of enzyme was done by homogenizing the apple in phosphate buffer and enzyme assay was performed by DNS method of (Miller, 1959). After extractions partially purification of crude enzyme extract by ammonium sulphate fractionation was carried out. The results of partial purification of amylase from apple are summarized (Table 1).

Figure 1: Elution Profile of Amylase on Sephadex G-100



Approximately 2.2 fold purification from homogenate was achieved during ammonium sulphate precipitation (50%-70%) with a yield of 40% enzyme where as in gel filteration chromatography 24 fold purification was achieved with specific activity of 4.76 U/mg (Table 1). The enzyme activity at 540 nm and protein content at 280 nm were determined for each fraction shown in Figure 1. The enzyme activity has been detected between the fractions No. 11 to the fraction No. 20, while other fractions had no enzyme activity.

Safey and Ammar (2004) reported approximately 5.66 fold purification of amylase from *Aspergillus Falvus var. columnaris* by ammonium sulphate precipitation 60% whereas 9.6 fold purification reported in case of gel filteration chromatography. Similarly (Shih *et al.*, 1995) reported approximately 20.5 fold purification of Amylase from *Clostridium perfringens* in case of gel filteration chromatography. Various fractions obtained during the study were subjected to NATIVE-PAGE to seek the homogeneity and purity of the enzyme (Figure 2).

Native Page

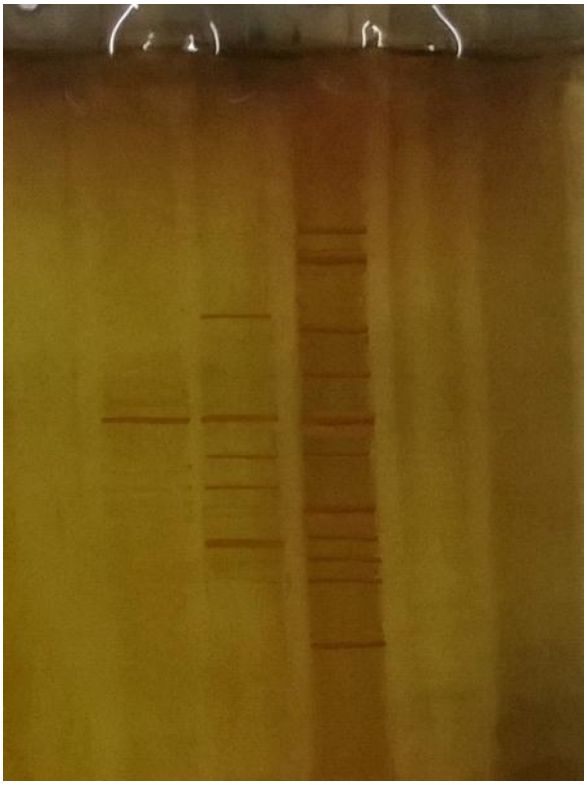
Temperature was also considered a important parameter to study the activity of amylase. Effect of temperature on

Table 1: Purification Summary of the Amylase Obtained from the Apple

S. No.	Purification Step	Volume	Total Enzyme Activity (units/ml)	Total Protein	Specific Activity (U/mg)	Purification Fold	Yield %
1	Crude Extract	1000	204	800	0.2	1	100
2	Ammonium Sulphate	200	81	200	0.4	2.2	40
3	Gel Filtration	20	11	2.4	4.76	24	11

Figure 2: Native Page-Lane 1: Purified Sample, Lane 2: Ammonium Sulphate Sample, Lane 3: Crude Extract

Lane 1, 2, 3



amylase activity was investigated in the range 25-50 ^{0}C. As the temperature increased from 20 ^{0}C to 35 ^{0}C enzyme activity increased. The optimum temperature for maximal Amylase activity was found to be 35 ^{0}C, after which the enzyme activity dropped (Figure 3). Likewise the optimum temperature for the alpha amylase obtained from *Malus pumila* was determined by keeping reaction mixture at various temperature ranges 5 ^{0}C to 75 ^{0}C and optimum temperature was 37 ^{0}C as reported by (Kanwal *et al.*, 2004). The pH dependence of amylase activity was assayed in a pH range of 4-11, using the standard reaction mixture. Enzyme showed the optimum activity at pH 6.0 (Fig.4) with activity of 4.6 U/mL. Effect of pH on alpha amylase purified from *Malus pumila* was determined by assaying enzyme at different pH ranging from 1-10 and amylase showed a pH optimum of 6.8 (Kanwal *et. al.*, 2004). For alpha amylase from *Penicillium olsonii* under the effect of some antioxidants vitamins, pH optimum was determined after incubation at different pH 3.6 to 6.8 at 30 ^{0}C for 30 minutes and maximum activity of enzyme was recorded at pH 5.6, and at 30 ^{0}C temperature (Affifi *et. al.*, 2008). Alpha amylase

Figure 3: Shows the Effect of Temperature on Enzyme Activity by Carrying Out Enzyme Reaction at pH 6 and at Different Temperature (25-50 °C), Each Value on the Graph Represents the Mean of Three Different Reading

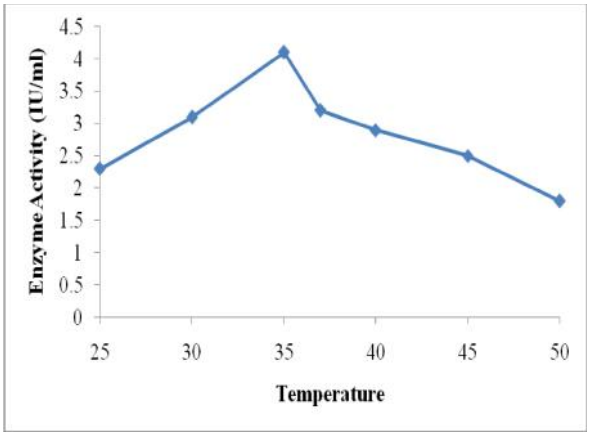
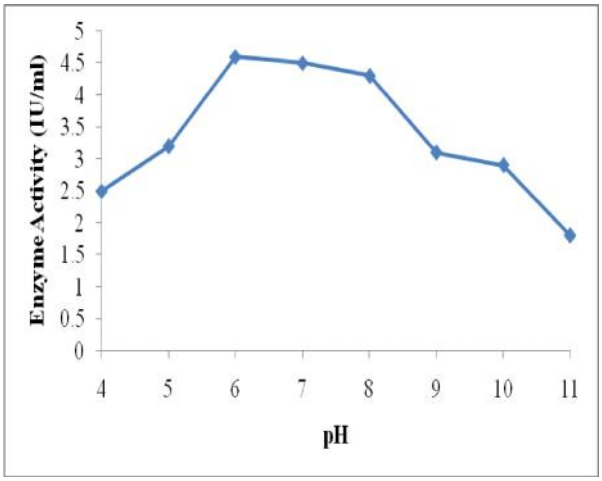


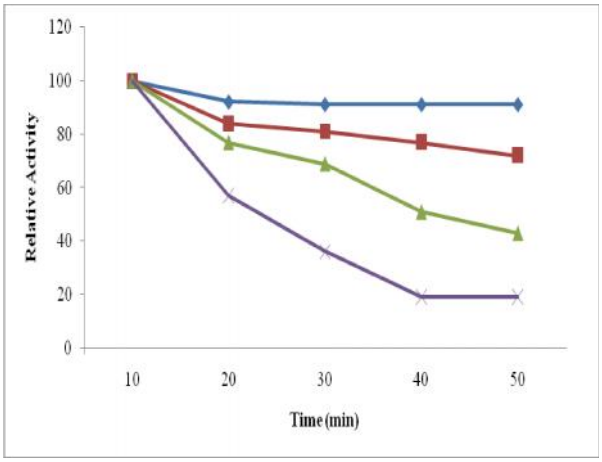
Figure 4: Shows the Effect of pH on Enzyme Activity by Carrying Out Enzyme Reaction at 35 °C at Different pH Value (4.0-11), Each Value on the Graph Represents the Mean of Three Different Reading



producing yeast strains such as *Saccharomyces cerevisiae* and *Saccharomyces kluyveri* exhibited maximum enzyme production at pH 5.0 (Samrat *et al*, 2011).

The addition of Ca^{+2}, Mn^{+2} and Mg^{+2} increased the enzyme activity A significant inhibitory effect on the protease activity was observed with Hg^{2+} (0% relative activity). Other metal ions which had a negative impact included Na^{+}, K^{+}, Cu^{+2}, Fe^{+2} and Zn^{+2}. Addition of Calcium chloride to the fermentation media increased the enzyme

Figure 5: Effect of Temperature on Stability of Amylase by Carrying Out the Enzyme Reaction at Different Temperature (40-70 °C), for (10-60 min) at pH 6.0, Each Value on the Graph Represents the Mean of Three Different Reading



production (Arthur *et al.*, 1996). Shih and Labbe (1995) showed in case of alpha amylase from *C. perfringes* that Ca²z presence provides stability to enzyme if used in 5mM concentration. Effect of metal ions and chemicals reagents was observed by (Chung *et al.*, 1995) when activity was measured at pH 5.5 at 70 °C in presence of various metal ions at 0.1 and 1mM and observed that thermo stability is enhanced in presence of the Ca²z. At 20 mM concentration of Hg²z completely inhibit the enzyme activity while Ba²z-33%, Cu²z-56%, and Zn²z- 36%. Time course of relative amylase activity at 40 °C, 50 °C, 60 °C and 70 °C is presented in Figure 5. The enzyme remained 80% active even after 1 h of incubation at 50 °C, while 90% activity at 40 °C and retained 45% activity after 1 h incubation at 60°C. However, activity declined to 15% at 70 °C.

Based on the above investigation it can be concluded that amylase from apple was partially purified and characterized and had maximal activity occurring at pH 6, and at temperature 35^0 and its activity was increased by the addition of metals like Ca^{+2} and Mn^{+2} and Mg^{+2} and it retains its activity near to stability at 40 °C upto 1 hour.

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