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Research Article

Spectrophotometric analysis of enzymatic profile of Carica papaya pectinesterase

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Abstract

Pectinesterase (PE) activity can be determined by several methods. Some determine the changes in the substrate (titration of the carboxyl groups, recording pH changes, colour change of pH indicator, gelation & viscosity and manometric assay), others measure the methanol liberated (colour reactions & titrations, gas chromatography and use of radioisotopes). Previous kinetics studies on the PE from Carica papaya have mostly used the titration method described. However, the present research attempts to assess the enzyme properties of papaya PE using a spectrophotometric method. PE isolated from papaya was investigated to determine its optimum pH and temperature. Optimum pH and temperature were estimated to be 7.4 and 75˚C, respectively. Kinetic parameters of the enzyme (Km and Vmax values) were evaluated by both titrimetric and spectrophotometric methods. The Km value of 3.06 mg/ml and Vmax of 2.2070 PE units/ml was obtained with the titration method. Whereas the spectrophotometric method gave a Km value of 3.43 mg/ml and Vmax of 0.0456 µmol/min. The effect of modulators on PE activity was also studied. It was observed that aluminium chloride and sodium bisulphite caused an increase and sodium bicarbonate decreased the Km value while calcium chloride and citric acid had negligible effect.

Keywords: *Carica papaya*, Pectinesterase, Enzyme kinetics, Titration method, Spectrophotometric Method, Modulators.

INTRODUCTION

Pectinesterase (PE) (EC 3.1.1.11) is a carboxylic acid esterase and belongs to the hydrolase group of enzymes (Alonso et al., 1995). It catalyzes the hydrolytic de-esterification of pectins causing pectin chains with a lower degree of esterification. PE in *Carica papaya* is a cell wall enzyme and plays a central role in the process of fruit softening during ripening. It is responsible for the changes taking place in the pectin composition of the fruits during ripening and processing. The enzymatic action of PE is responsible for the process of gel formation shortly after the papaya is pureed (Yamamoto & Inouye, 1963). Hence, PEs are widely used in clarification of fruit juices and to prevent increased water retention caused by gelation of juices. The control of PE activity, through the knowledge of its dependence on temperature and pH, is of great practical importance in the food industry for protecting and improving the texture and firmness of several processed fruits and vegetables (Castaldo et al., 1989). Thus, PEs potentially affects the quality of the finished processed products (Versteeg et al., 1978).

PE from different sources shows different kinetic properties also, PEs from different varieties of the same fruit differ in their properties (Pressey & Avants, 1972). The isoelectric points of plant PEs were always above pH 7 and the pH optimum ranged between 6 and 8.5. The values of K_m for plant PEs vary widely from 0.0046 to 2.3 mg/ml and vary even within the different varieties of the same fruit (Fayyaz

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et al., 1993). This difference may be related to the degree of purity of the enzyme and substrate but it seems that these differences are most probably due to the varietal differences of the fruit because these enzymes also differ in other properties such as response to temperature, pH and NaCl concentrations for their optimum activity (Foda et al., 2016).

Plant PE is competitively inhibited by pectate, one of its own reaction products (Termote et al., 1977). Cations are well known to affect the activity of PE when pectin is used as the substrate (Rexova-Benkova & Markovic, 1976). The divalent cation like Mg^{2+} increase the activity of papaya PE more markedly than the monovalent cations like K^+ and Na^+ and K^+ is more stimulatory than Na^+ (Lim & Chung, 1993). Glycerol, glucose, and sucrose were non-competitive inhibitors, while the corn syrups were uncompetitive inhibitors of papaya PE (Chang et al., 1965). The inhibition by sugars and alcohols is ascribed to the reduction of the water activity (Lee & Wiley, 1970). Papaya PE from the variety *exotica* is much more strongly inhibited by polygalacturonic acid (competitive inhibitor) than PE from papaya variety *solo* Fayyaz et al., 1994). Alginic acid shows a mixed type of inhibition termed competitive-non-competitive inhibition on papaya PE, because the pattern observed lies between those for competitive and non-competitive inhibition (Fayyaz et al., 1994). PE from other plants is inhibited by polygalacturonic acid and it acts as a competitive inhibitor (Lee & Macmillan, 1968); (Lourenco & Catutani, 1984).

Several methods are available to determine the PE activity. Some determine the changes in the substrate (titration of the carboxyl groups, recording pH changes, colour change of pH indicator, gelation & viscosity and manometric assay), others measure the methanol liberated (colour reactions & titrations, gas chromatography and use of radioisotopes). The increase in viscosity of pectin solutions has been used to measure PE quantitatively (Weurman, 1954). The activity of PE can be followed by titration of the liberated carboxyl groups with diluted (0.1-0.002 mol/1) alkali PE activity can be measured by direct recording of the pH drop over a short pH interval in an unbuffered substrate solution (Somogyi & Romani, 1964). A quantitative method to estimate the PE activity in which the colour change of a pH indicator is measured with a spectrophotometer was used (Brady, 1976). A suitable pH indicator such as methyl red or bromothymol blue with pectin in test tubes can be used as a sensitive qualitative screening method for PE activity. Recently, PE assays are commonly based on spectrophotometry using chemical (Seymour et al., 1991). Enzymic derivatisation (Klavons & Bennett, 1986); (Mango & Haas, 1997); or on spectrofluorimetry with enzymic derivatisation (Wojciechowski & Fall, 1996). Previous kinetics studies on the PE from papaya have mostly used the titration method described. However, the present research attempts to assess the enzyme activity of papaya PE using a spectrophotometric method.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used were of Analytical Grade and obtained from Loba chemie, Merck and Sigma Chemical Company. They were used without further purification.

Plant Material

Carica papaya fully ripened fruits with firm texture and without bruises or damage were purchased from local market Mumbai, India on need basis. After properly washing with distilled water, they were peeled to remove the skin and the fruit pulp was used for the enzyme extraction.

Enzyme Extraction

The extraction of the enzyme was carried out by homogenizing 5 grams of the ripe fruit pulp with chilled 2 M NaCl solution. The homogenate was filtered through cotton and the volume of the filtrate was then made up to 100 ml with the same chilled 2 M NaCl solution. This 5 gm crude enzyme extract was used to estimate the optimum pH, the optimum temperature and study the enzyme kinetics of PE from papaya.

Optimum pH and Optimum Temperature for PE

The spectrophotometric method as described by (Hagerman & Austin, 1986). With modifications was followed to determine the optimum pH and optimum temperature of the papaya PE. Briefly, the optimum pH of the PE extract was estimated by incubating a mixture of 10 ml of 1 gm % pectin substrate 1 ml of 0.2 M phosphate buffer of varying pH range from 6.2 to 8.0 and 1 ml of 1% bromothymol blue dye with 1 ml of the 5 gm% enzyme extract. After the completion of one hour incubation period at room temperature, the tubes were read calorimetrically at 420 nm against the substrate blank. Non-enzymatic de-esterification was determined at each pH value in the absence of the PE enzyme and corrections were made in the absorbance values of the tubes containing enzyme. The plot of corrected absorbance values against the varying pH was used to estimate the optimum pH of PE. Similarly, the optimum temperature of the PE enzyme was estimated by carrying out the enzyme assay at pH 7.4 and temperatures between 25°C and 85°C.

Enzyme kinetics

The activity of PE as a function of the pectin concentration was measured by following two different methods. First was the titration method as described by (Korner et al., 1980). Briefly, the reaction mixture consisted of varying aliquots (3, 6, 9, 12 and 15 ml) of 1 gm% pectin, 1 ml of the crude PE extract, 1 ml of the 0.2 M phosphate buffer of

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pH 7.4 and distilled water to make up to the final volume of 17 ml in all the conical flasks. The flasks were incubated for an hour in a water bath set at 75°C with intermittent shaking. After cooling the flasks under tap water, the contents of the flask were titrated against 0.02 M NaOH in presence of phenolphthalein indicator. The enzyme activity was calculated using the formula given below. One unit of PE was defined as the amount of enzyme which produced l µEq of acid per min.

$$\text{PE units/ml} = \frac{(\text{Volume of NaOH})(\text{Molarity of NaOH})(1000)}{(\text{Time in minutes})(\text{Volume of enzyme})}$$

The second was the spectrophotometric method using bromothymol blue dye as described by (Hagerman & Austin, 1986) with modifications. Precisely, 1 gm pectin in varying aliquots (3, 6, 9, 12 and 15 ml), 1 ml of the 0.2 M phosphate buffer of pH 7.4, 1 ml of the 1% bromothymol blue dye and distilled water was added to make up the final volume to 17 ml in all the sugar tubes. The substrate blank consisted of all the reagents as the test except the pectin which was replaced with 15 ml of distilled water. Lastly, 1 ml of the crude PE extract was added to all the tubes including blank. All the tubes were vortexed and incubated for an hour in a water bath maintained at 75°C. The reaction was stopped by cooling the tubes on ice and 5 ml distilled water was added just before the tubes were read calorimetrically against the substrate blank. The absorbance values at 420 nm were plotted against the varying pectin concentration to calculate the Michaelis constant (Km).

Effect of Modulators on PE Activity

The effect of modulators on the PE activity was studied by following the spectrophotometric method described above. The modulators considered for the study included aluminium trichloride, ascorbic acid, calcium chloride, sodium bisulphite, sodium bicarbonate and sodium carbonate of 0.05 M concentration each. Briefly, in the sugar tube 9 ml of 1 gm pectin and 1 ml each of the 0.2 M phosphate buffer of pH 7.4, the modulator and 1% bromothymol blue dye were added. The total volume was made up to 17 ml with distilled water. The substrate and the modulator volume in the blank were replaced with 10 ml of distilled water. Lastly, 1 ml of the crude PE extract was added to all the tubes including the blank. After overtaxing all the tubes were incubated in a water bath of 75°C temperature for an hour. The tubes were cooled on ice to stop the reaction and were read calorimetrically against the substrate blank. The effect of the modulators on the PE activity was estimated from the Line weaver-Burk plot.

RESULTS AND DISCUSSION

Pectolytic enzyme is well extracted with NaCl than with water (Pozsar-Hajnal & Polacsek-Racz, 1975) and their extraction is affected by the concentration of NaCl used (Fayyaz et al., 1993). Reported that PE obtained from papaya gave maximum enzyme activity when it was extracted using 2 M NaCl. Thus, in the present study the extraction of PE from papaya was carried out using 2 M NaCl.

Optimum pH and Optimum temperature for PE

The results obtained for the effect of pH on the enzyme activity of papaya PE are presented in Figure 1. The enzyme activity increased from pH 6.2 to 7.4 where the maximum activity was recorded at pH 7.4 and thereafter it decreased. Thus, the optimum pH of 7.4 determined in this study was closer to the value of 7.5 reported by (Chang et al., 1965) for papaya PE. However, (Fayyaz et al., 1995) and (Lourenco & Catutani, 1984). Reported an optimum pH of 8.0 where as reported optimum pH of 7.0 for the papaya PE.

The effect of temperature on the activity of papaya PE was studied over a temperature range of 25-85°C while the pH of the reaction mixture was kept constant at 7.4. The findings for the same are illustrated in **(Figure 2)**. The rate of enzyme reaction increased from 25°C to 75°C and declined thereafter. Thus, the maximum enzyme reaction was observed at 75°C. This optimum temperature of 75°C for papaya PE differed from those reported by (Lourenco & Catutani, 1984). (60°C), (65°C) and (55°C) by (Fayyaz et al., 1995).

The differences in the optimum conditions of pH and temperature could be attributed to the variance in the properties of PE obtained from different varieties of papaya as reported by (Fayyaz et al., 1994).

Enzyme Kinetics of PE

The effect of pectin concentration on the activity of papaya PE was studied. The substrate for the 5 gm crude enzyme extract of papaya PE was apple pectin. The determination of the Michaelis-Menten constant (K_m) for the enzyme was carried out following two different methods. The results of the double reciprocal plot for the titration method and the spectrophotometric method are presented in **(Figures 3 and Figure 4)**.

The K_m value of 3.06 mg/ml and V_{max} of 2.2070 PE units/ml was obtained with the titration method. Whereas the spectrophotometric method gave a K_m value of 3.43 mg/ml and V_{max} of 0.0456 µmol/min. The K_m value for papaya PE reported from earlier studies was 0.3 mg/ml (Chang et al., 1965). 0.12 mg/ml , 0.11 mg/ml (Fayyaz et al., 1995) and 3.2 mg/ml The purification of papaya PE yielded two forms of the enzyme designated as PME 1 (Km = 0.0071 mg/ml) and PME 2 (Km = 0.0166 mg/ml) with optimum pH above 9 and optimum temperature of 35°C (Lim & Chung, 1993).

Effect of Modulators on PE

The kinetics of enzymatic reactions can be considerably modified by the presence of modulators in the reaction

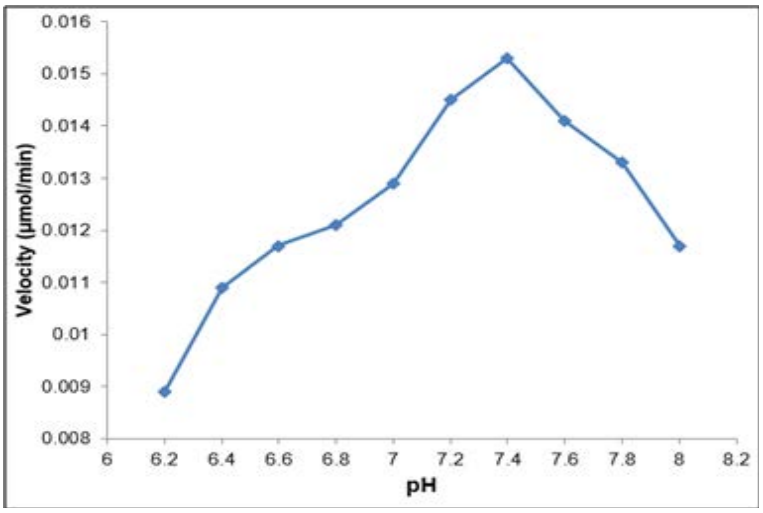

Figure 1. Effect of pH on enzyme activity of papaya PE.

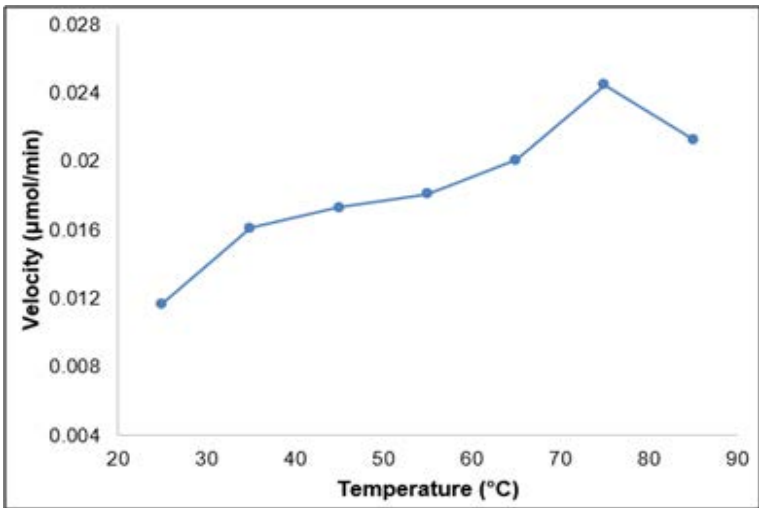

Figure 2. Effect of temperature on enzyme activity of papaya PE.

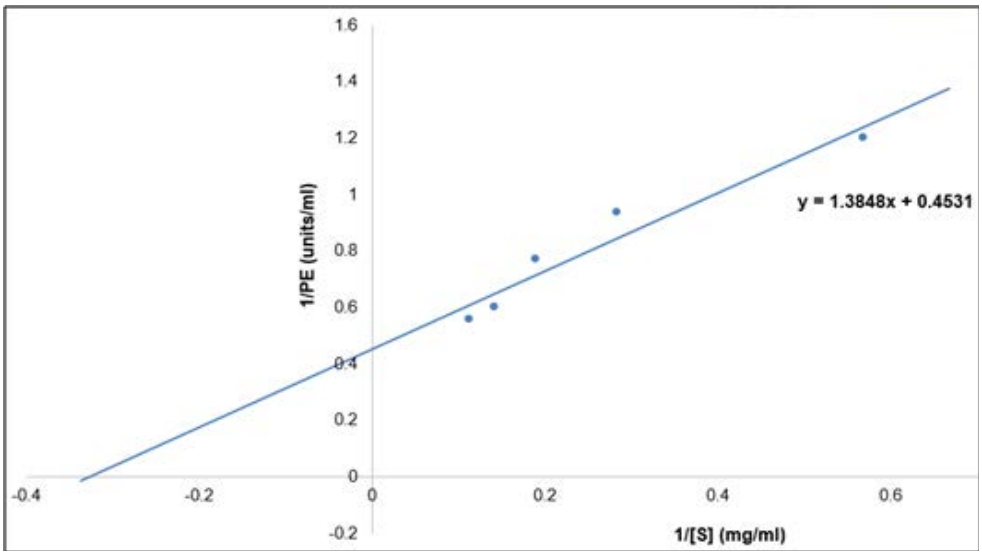

Figure 3. Double reciprocal plot of papaya PE by titration method of (Kertesz, 1995).

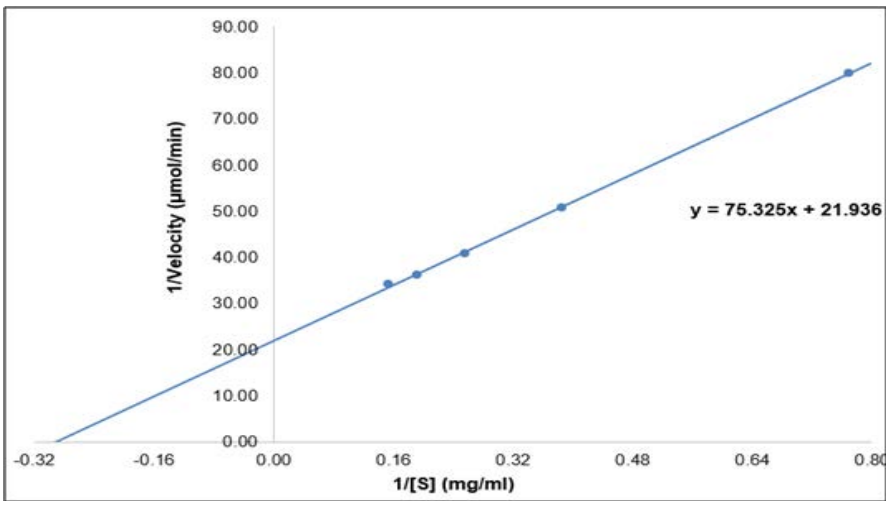

Figure 4. Double reciprocal plot of papaya PE by spectrophotometric method of (Hagerman & Austin, 1986).

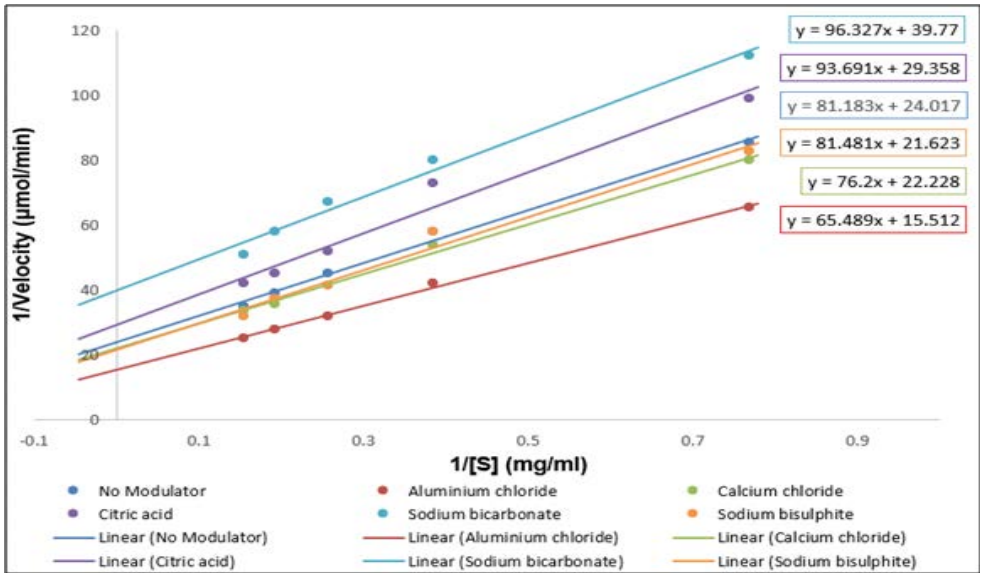

Figure 5. Effect of modulators on the activity of papaya PE.

Table 1. Effect of modulators on the activity of papaya PE.

Modulators	Linear Equation	K_m	V_{max}
		(mg/ml)	(µmol/min)
Control (No modulator)	y = 81.183x + 24.017	3.38	0.0416
Citric acid	y = 93.691x + 29.358	3.19	0.0341
Aluminium chloride	y = 65.489x + 15.512	4.22	0.0645
Calcium chloride	y = 76.2x + 22.228	3.43	0.0450
Sodium bicarbonate	y = 96.327x + 39.77	2.42	0.0251
Sodium bisulphite	y = 81.481x + 21.623	3.77	0.0462

medium. Those modulators which decrease the enzymatic reactions are known as inhibitors while modulators can also cause an increase in the enzymatic reactions and thus are referred to as the activators of the enzyme. The study of enzyme kinetics in presence of modulators is often used to obtain information about the mechanism of enzyme action. Thus, the effect of organic modulator-citric acid and inorganic modulators like anhydrous salts of aluminium chloride ($AlCl_3$), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), and sodium bisulphite ($NaHSO_3$) on papaya PE was studied and the results are illustrated in **(Figure 5 and Table 1)**.

The presence of aluminium chloride and sodium bisulphite resulted in an increased activity of papaya PE while sodium bicarbonate exhibited an inhibitory effect. The calculated K_i value for sodium bicarbonate was 2.42 mg/ml. The effect of calcium chloride and citric acid on papaya PE activity was negligible (Delincee & Radola, 1970).

CONCLUSION

Carica papaya PE under study differed in its requirement for optimum conditions as well as the K_m value compared to that of the earlier reports on this enzyme from papaya. These variances could be attributed to the differences in the variety of papaya, geographic location, and climatic conditions. Further, spectrophotometric method was successfully used to study the properties of Pectinesterase isolated from papaya. The ability to judge the end-point of the visual titration differs among the individuals and is susceptible to human error. The results obtained using titration method by different individuals varies. Therefore, the spectrophotometric method could be a better alternative for the enzymatic study of Pectinesterase.

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