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Stability studies of papaya pectinesterase

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Stability studies were carried out on the purified papaya (*Carica papaya* L. var. *exotica*) pectinesterase (EC 3.1.1.11). The enzyme preparation was stable at 4°C in 0·02M sodium phosphate buffer (pH 7·5) solution containing 0·2M NaCl and 0·02% sodium azide and the loss in activity was less than 5% after storage for 1 year. The thermostability studies showed that enzyme was more heat-stable at pH 7·5 than at pH 4. After heating at 60°C for 5 min, 65·6 and 82% activity still remained at pH 4 and pH 7·5, respectively. It was completely inactivated by heating for 5 min at 70 and 75°C at pH 4 and pH 7·5, respectively. The *D* values (time to inactivate 90% of the enzyme) at 55, 60, 65 and 70°C at pH 4·0 were estimated to be 112·14, 23·78, 8·33 and 1·71 min, respectively. Lower inactivation rates were observed for pH 7·5, with the *D* values ranging from 143·27 to 1·67 min for temperatures between 60 and 75°C. The *Z* values, which indicate the rise in temperature necessary to observe a 10-times faster rate of heat-inactivation, were estimated to be 7·8°C and 8·38°C at pH 7·5 and pH 4·0, respectively. The inactivation energies and Q_{10} values were calculated as 256·9 kJ/mol and 15·59 at pH 4·0 and 284·76 kJ/mol and 19·21 at pH 7·5, respectively. pH stability studies showed that the enzyme was stable from pH 4–11 after exposure of the enzyme to these pHs for 24 h at 30°C. More than 85% of the activity was retained in all of these cases. However, at pH 1 and 12, the enzyme was unstable and it completely lost its activity after 24 h of incubation at 30°C.

INTRODUCTION

Papaya pectinesterase (EC 3.1.1.11) is a pectic enzyme which has an important influence on the quality and stability of processed papaya products. A short period after papaya is pulped into puree, a gel is formed. This gel formation has been attributed to the enzymatic action of pectinesterase (Yamamoto & Inouye, 1963). Pectinesterase is also of great concern to the citrus industry since it has been definitely established that it is the causative agent for clarification of citrus juices and gelation of concentrates (Joslyn & Pilnik, 1961; Krop, 1974). According to Versteeg *et al.* (1980), to prevent these quality defects in the citrus industry, the enzyme must be inactivated by pasteurisation at 90°C for 1 min. On the other hand, the heat treatment necessary to inactivate the pectinesterase in the juice varies with variety, pH, etc. (Rouse & Atkins, 1952, 1953; Atkins & Rouse, 1953; Kew *et al.*, 1957). Eagerman and Rouse (1976) examined the pectinesterase inactivation in juices extracted from different citrus varieties and found that the *Z* values (the rise in temperature necessary to observe a 10-times faster heat-inactivation) varies depending on the variety. Nath and Ranganna (1977) showed that the processing time for pectinesterase inactivation increased as the pH increased. Low pH inactivation of pectinesterase has been suggested as an alternative to pasteurisation in citrus juice (Owusu-Yaw *et al.*, 1988).

This work was designed to carry out stability studies (including storage stability, thermostability and pH stability) on purified papaya (var. *exotica*) pectinesterase.

MATERIALS AND METHODS

Enzyme source

Previously extracted and purified to homogeneity, papaya (*Carica papaya* L. var. *exotica*) pectinesterase, was used in this study (Fayyaz *et al.*, 1993; Fayyaz *et al.*, 1994*a,b*). The concentrated enzyme preparation had a specific activity of 780 μmol/min/mg protein (500 units per ml of enzyme solution). The enzyme was kept in 0·02 M sodium phosphate buffer (pH 7·5) solution containing 0·2M NaCl and 0·02% sodium azide at 4°C.

Enzyme assay

The pectinesterase activity was determined by the method of Kertesz (1955), as described by Korner *et al.*

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(1980). Briefly, the method consisted of a titrimetric measurement of the rate of carboxyl group liberation from 1% pectin, 0·15M NaCl solution at pH 7·0 and 30°C. The initial reaction velocity was measured by automatic titration of the liberated carboxyl groups with 0·02 M sodium hydroxide for 10 min in a Titralab Autotitrator model VIT 90/ABU 93/SAM 90 (Radiometer, Copenhagen, Denmark). Non-enzymatic de-esterification of pectin was determined in the presence of denatured pectinesterase and corrections were made to obtain the correct rate of the enzyme reaction. One pectinesterase unit is defined as the activity corresponding to the release of one μmol of carboxyl group per minute.

Storage stability studies

Concentrated enzyme (having 0·6416 mg/ml protein) was stored in 0·02M sodium phosphate buffer (pH 7·5) containing 0·2M NaCl and 0·02% sodium azide at 4°C. Residual activity was tested after 1 year storage under standard assay conditions.

Thermostability studies

Residual activities of pectinesterase after incubation for 5 min at various temperatures at pH 4 and 7·5 were determined. Citric acid–phosphate buffer (0·1 M) containing 0·02% sodium azide was used in these studies. Tubes that were identical in length, diameter and weight were used in all of these studies. Pectinesterase (15 μl) solution was added and mixed in the pre-incubated screw-type tubes having 1485 μl of buffer solution at the desired temperature in a circulating waterbath. After incubating the tubes for 5 min at the desired temperatures, the tubes were cooled in the water with melting ice and, immediately after that, residual activity was measured under standard assay conditions.

Table 1. *D* values (rates of inactivation) in minutes at pH 4 and 7·5

Temp. (°C)	Slope at pH 4	*D* value at pH 4	Slope at pH 7·5	*D* value at pH 7·5
55	−0·00891	112·14	—	—
60	−0·03652	27·38	−0·00698	143·27
65	−0·12008	8·33	−0·03248	30·79
70	−0·58374	1·71	−0·13471	7·42
75		—	−0·59705	1·67

D value = 1/slope.

***D* and *Z* values**

The rate of heat-inactivation of purified papaya pectinesterase were determined from 55 to 75°C in pH 4 and 7·5 citric acid–phosphate buffer containing 0·02% sodium azide as an antimicrobial agent. Pectinesterase solution (80 μl) was added to the identical test tubes containing 7920 μl of buffer and incubated at the desired temperature in a circulating water bath. One ml samples were taken out at various time intervals and cooled in water with melting ice. Residual activities were determined immediately after the samples were cooled down. To calculate the *D* values (decimal reduction value which is the time period required at a certain temperature to reduce the activity to 10%), the log of % residual activity was plotted against heating time. *Z* values (the rise in temperature necessary to observe a 10-times faster heat-inactivation) were calculated by plotting the log of *D* values against temperature (°C) (Table 1).

Inactivation energy and Q_{10} values

To determine the inactivation energy, *K* was calculated from the data for *D* values (Tables 2 and 3) and $L_n{\cdot}K$ was plotted against the absolute temperature (K). ΔH was calculated from the slope by formula, E_a = slope × *R*. Inactivation energy and Q_{10} for inactivation energy was calculated as described by Toledo (1991).

Table 2. Rate constants at different temperatures for the calculation of inactivation energy at pH 4

Temp. (°C)	Temp. (°K)	1/*T* (°K)	*D* value (min)	$K = 2{\cdot}303/D$ (min^{-1})	L_nK
55	328	0·003408	112·14	0·020536	−3·88553
60	333	0·003003	27·38	0·084112	−2·47560
65	338	0·002958	8·33	0·276470	−1·28565
70	343	0·002915	1·71	1·346783	−0·29772

D value = 1/slope.

Table 3. Rate constants at different temperatures for the calculation of inactivation energy at pH 7·5

Temp. (°C)	Temp. (°K)	1/*T* (°K)	*D* value (min)	$K = 2{\cdot}303/D$ (min^{-1})	L_nK
60	333	0.003003	143.27	0.016074	−4.13051
65	338	0.002958	30.79	0.074797	−2.59297
70	343	0.002915	7.42	0.310337	−1.16996
75	348	0.002873	1.67	1.379041	−0.32139

D value = 1/slope.

pH stability studies

Purified papaya pectinesterase was incubated at 30°C for 24 h at pH 1–12. Four different buffer solutions were used to cover this range of pH in these studies. Residual activities of each preparation were assayed under standard assay conditions. Rate of pH inactivation of purified papaya pectinesterase were also determined from pH 1–12 at 30°C. After preliminary studies two sets of experiments were designed. In the first set of experiments (pH 2·2–11), rates of inactivation were determined for 5 days and in the 2nd set (pH 1, 1·5, 11·5 and 12), rates of inactivation were determined up to 100 min. For pH 2·2–11, enzyme solution (70 μl) was incubated in the 3430 μl of respective buffer solution containing 0·02% sodium azide at 30°C. Samples of 500 μl were taken out after 24 h, and, immediately after that, residual activity was determined under standard assay conditions. For pH 1, 1·5, 11·5 and 12, samples were incubated for 100 min in the respective buffers containing 0·02% sodium azide and samples were taken after different intervals and residual activity was measured immediately under the standard assay conditions.

RESULTS AND DISCUSSION

Papaya (var. *exotica*) pectinesterase was stable at 4°C in 0·02M sodium phosphate buffer (pH 7·5) solution containing 0·2M NaCl and 0·02% sodium azide and the loss in activity was less than 5% after storage for 1 year. This stability was as good as the pectinesterase from other fruits. Loss of activity of purified pectinesterase from navel orange in solution of 0·005 mol/litre sodium phosphate, pH 7·5 with 0·1 mol/litre sodium chloride was less than 15% after storage for 2 years at 4°C (Versteeg, 1979). On the other hand, pectinesterase purified from jelly fig achenes was stable in distilled water or in 0·2 M NaCl for 6 months at room temperature (Lin *et al.*, 1989).

The thermostability studies on papaya pectinesterase were carried out at pH 4 and pH 7·5 at different temperatures. pH 4 was selected because it was the operational pH for papaya puree processing. pH 7·5 was selected, first because the heat-stabilities of purified pectinesterases from different sources had been determined at pH 6–7·5 (Pressey & Avants, 1972; Nakagawa *et al.*, 1970; Manabe, 1973; Castaldo *et al.*, 1989; Seymour *et al.*, 1991) and second to compare the results with Manabe (1973) and Castaldo *et al.* (1989), since they have determined the heat-stabilities at the same pH. Secondly, pH 7·5 was selected to see the difference in inactivation at different temperatures due to the variation in pH since, according to Nath and Ranganna (1977), the processing time for pectinesterase inactivation increased as the pH increased. In the first part of this study, heat-stability of the enzyme was determined after heating it for different temperatures at pH 4 and pH 7·5 for 5 min and the results are shown

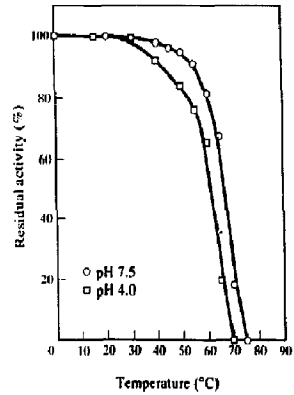

Fig. 1. Effect of temperature on the stability of papaya pectinesterase.

in Fig. 1. According to the results, the enzyme was more heat-stable at pH 7·5 than at pH 4. It was completely inactivated by heating for 5 min at 70 and 75°C at pH 4 and 7·5, respectively. About 82% of enzyme activity still remained after incubation at 60°C, pH 7·5 for 5 min, but at pH 4, after 5 min incubation at 60°C, a value of 65·6% for residual activity was recorded.

According to published data, the temperature of inactivation of plant pectinesterases covers a wide range. Heating for 5 min at 65°C gave complete inactivation of purified tomato pectinesterase at pH 7 (Nakagawa *et al.*, 1970), whereas, on the other hand, purified citrus natsudaidai pectinesterase was completely inactivated after 5 min incubation in 0·01 M potassium phosphate buffer (pH 7·5) solution at 80°C (Manabe, 1973). A large difference in thermostability was observed between thermolabile pectinesterase (TLPE) and thermostable pectinesterase (TSPE), purified from marsh white grape fruit pulp by Seymour *et al.* (1991). They tested the thermostability of both enzymes by heating them for 5 min at various temperatures from 40 to 85°C at pH 7 in 10 mM phosphate buffer and measured the residual activity. TLPE and TSPE were completely inactivated at 65 and 85°C, respectively. The only report available on the thermostability of papaya (var. *solo*) pectinesterase by Lourenco and Catutani (1984) shows that about 50% of the enzyme activity remained after incubating at 65°C for 5 min. pH of the incubation medium was not mentioned but most probably it was 7·5, because the enzyme was stored in 5 mM potassium phosphate buffer (pH 7·5) at −20°C. On the other hand, the enzyme purified in this study from papaya (var. *exotica*) showed 67·8% residual activity after incubating it at pH 7·5 and 65°C. Results obtained from the above experiment are also in agreement with the results of Joslyn and Sedkey (1940). They found a marked difference in thermal stability of pectinesterase in citrus juices according to species and varieties. Pressey and Avants (1972) have also shown

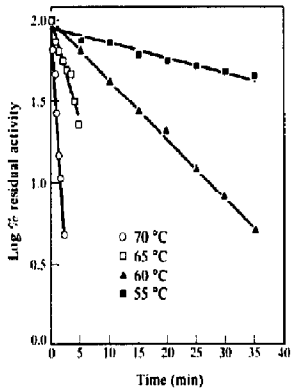

Fig. 2. Thermal inactivation rates of purified papaya pectinesterase at pH 4·0.

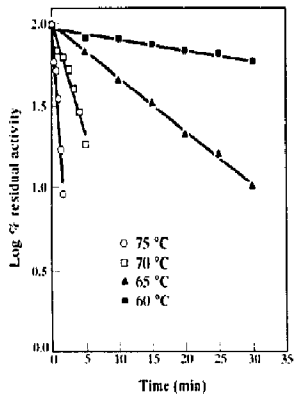

Fig. 3. Thermal inactivation rates of purified papaya pectinesterase at pH 7·5.

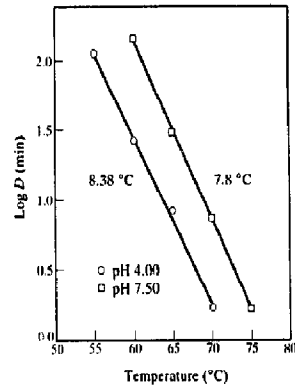

Fig. 4. Thermal destruction curves for purified papaya pectinesterase.

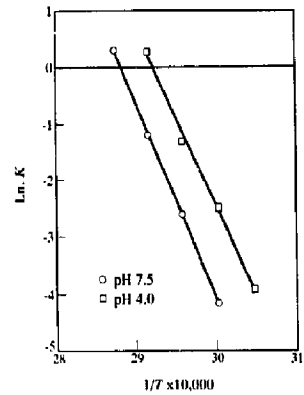

Fig. 5. Graphic method for the calculation of inactivation energy of papaya pectinesterase.

that the pectinesterases purified from different varieties of tomato were different in terms of stability to heat.

In the next experiment the rates of heat-inactivation of purified papaya pectinesterase were determined at pH 4 and 7·5 at different temperatures (Figs 2 and 3). Citric acid–phosphate buffer was used in these studies. D values at 55, 60, 65 and 70°C in pH 4 buffer solution were 112·14, 27·38, 8·33 and 1·71 min, respectively (Table 1). Lower inactivation rates were observed at pH 7·5 and D values were calculated as 143·27, 30·79, 7·42 and 1·67 min at temperatures 60, 65, 70 and 75°C, respectively. These results are in agreement with those of Nath and Ranganna (1977) who showed that the processing time for pectinesterase inactivation increased as the pH increased. Increasing the pH from 4 to 7·5 at 70°C increased the D value by approximately 4·4-fold. At 70°C a D value of 1·71 min occurred for the inactivation of purified pectinesterase at pH 4 while a D value of 7·42 min resulted when the enzyme was inactivated at pH 7·5.

From these D values the changes in temperature required to increase the inactivation rate 10-fold (Z value), were calculated to be 7·8 and 8·38°C at pH 7·5 and pH 4, respectively, as shown in Fig. 4. These values lie in the range observed by Versteeg *et al.* (1980) for purified pectinesterases from navel orange. They reported Z values of 6·5°C for pectinesterase I and pectinesterase III and a Z value of 11°C for pectinesterase II at pH 4. In another report Wicker and Temelli (1988) have shown Z values of 10·8 and 6·5°C for the sensitive and stable fractions from Valencia orange pectinesterases.

Inactivation energies were calculated from the data obtained for D values given in Tables 2 and 3. The values were calculated to be 257 kJ/mol and 285 kJ/mol at pH 4 and 7·5, respectively, as shown in Fig. 5. Q_{10} values for inactivation energy were calculated as 15·59 and 19·21 at pH 4 and 7·5, respectively.

Comprehensive pH stability studies were carried out on this purified papaya pectinesterase, which included

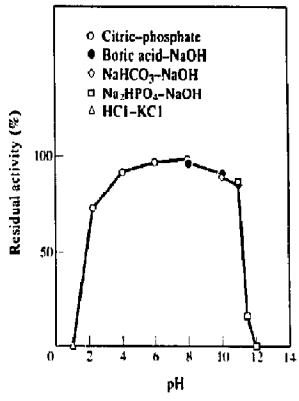

Fig. 6. Effect of pH on the stability of papaya pectinesterase.

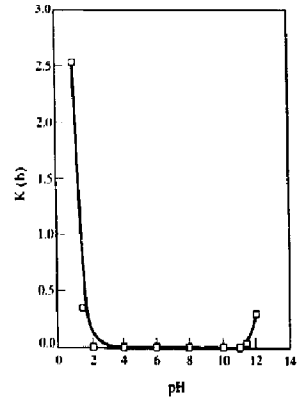

Fig. 9. Schematic representation of effect of pH on the stability of purified papaya pectinesterase.

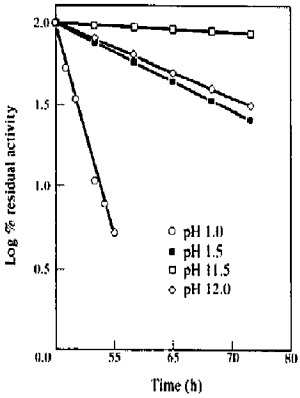

Fig. 7. pH inactivation rates of purified papaya pectinesterase at 30°C.

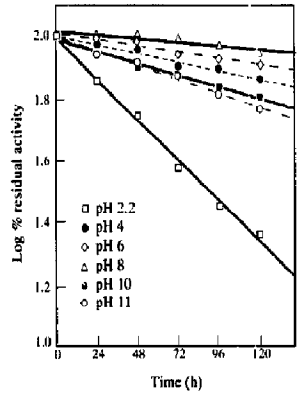

Fig. 8. pH inactivation rates of purified papaya pectinesterase at 30°C.

pH stability for 24 h at 30°C in the pH range 1–12 and measurements of rates of inactivation of the enzyme incubated at different pHs. As shown in Fig. 6, the 100% enzyme activity remained at pH value 8. In contrast, pectinesterase activity decreased under both acidic and alkaline conditions. The enzyme was stable at pH 4–11 and it retained more than 85% activity, but at pH 1 and 12 the enzyme was unstable and it completely lost its activity after 24 h incubation at 30°C. On the other hand, purified pectinesterase from *Ficus awkeotsang* has shown different types of response to pH stability (Komae *et al.*, 1990). This enzyme was incubated at 25°C for 24 h at different pHs. All the enzyme activity remained at pH values of 8–11 and decreased under the slightly acidic conditions in the pH range 5–7 and more than 50% of the enzyme activity was lost at pH 4 and 12. Enzyme purified in this study from papaya (var. *exotica*) was more stable at pH 4 and less stable at pH 12 as compared to the *Ficus awkeotsang* pectinesterase purified by Komae *et al.* (1990). In another report by Manabe (1973), citrus natsudaidai pectinesterase was unstable at pH 3 and 10 and was completely inactivated within 10 min. At pH ranges from 5 to 8, however, it was considerably stable. These differences in stability may be related to its high

Table 4. Effect of pH on the rate constant (slope)

pH	Rate constant (h)
1·0	−2·525840
1·5	−0·348570
2·2	−0·005449
4·0	−0·001110
6·0	−0·000800
8·0	−0·000468
10·0	−0·001507
11·0	−0·003258
11·5	−0·033000
12·0	−0·300640

incubation temperature because natsudaidai pectinesterase was incubated at 50°C and the temperature of incubation is an extremely critical parameter in determining the stability of an enzyme.

The next attempt was made in order to get the complete stability data as described by Whitaker (1972) and the results are shown in Figs 7–9 and in Table 4. Figures 7 and 8 show the rates of inactivation of the purified papaya pectinesterase incubated at different pH values at 30°C. These figures show that the loss in activity was due to the denaturation of the enzyme because, according to Whitaker (1972), when pH affects only denaturation of an enzyme, plots of log (% activity left) versus time will usually be straight lines. The slopes of the lines are the first order rate constants for loss of enzyme activity. In Fig. 9, rate constants obtained from Figs 7 and 8 are plotted against pH. The enzyme was stable over a pH range of 4–11 and became unstable at pH 12 and more unstable at pH 1.

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