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Purification and properties of pectinesterase from soursop (*Anona muricata*) pulp

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Two forms of pectinesterase were purified using the techniques of ammonium sulphate fractionation, ion-exchange chromatography and gel filtration. PE I had a specific activity of approximately 4 units mg^{-1} (43-fold), that of PE II was 6.4 units mg^{-1} (229-fold). These pectinesterases (PE I and PE II) had approximate molecular weights of 29 100 and 24 100, respectively, as estimated by gel filtration, and 31 000 and 28 000, respectively, as estimated by sodium dodecyl sulphate polyacrylamide electrophoresis. The optimum temperature for enzyme activity was shown to be 60 °C for both PE I and PE II. The activation energies of PE I and PE II were calculated as 36 kJ mol^{-1} and 42 kJ mol^{-1}, respectively. The optimum pH values for both pectinesterases lie within the range 7.0–8.0. The K_m value for PE I was 0.52 mg ml^{-1} and 0.0843 mg ml^{-1} for PE II. PE I had a maximum velocity (V_{max}) of 154 μmol mg^{-1} min^{-1}, and PE II a V_{max} of 726 μmol mg^{-1} min^{-1}.

INTRODUCTION

In food technology, the presence of pectinesterase in citrus fruit is a problem that has been intensively studied, particularly in relation to cloud loss in citrus juice (Pilnik & Voragen, 1991). Furthermore, biochemical characterization of pectinesterase has been conducted in a number of fruits and plant tissues, including ripe peach (Glover & Brady, 1994), tomato (Giovane *et al.*, 1994; Warrilow *et al.*, 1994), lemon (MacDonald *et al.*, 1993) and mung bean hypocotyl cell walls (Bordenave & Goldberg, 1993). The enzymes purified from these different sources usually exhibit different molecular and kinetic properties. This paper describes the purification of pectinesterase from soursop pulp and some of the properties of the enzyme.

MATERIALS AND METHODS

Materials

Citrus pectin was purchased from Fluka (Buchs, Switzerland). Its degree of esterification was found to be 63–66%. Sephadex G-100 and CM-Sephadex C-50 were obtained from Sigma (St. Louis, MO, USA). Molecular weight markers were obtained from Pharmacia (Milton Keynes, UK). All other chemicals were reagent grade.

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Ripe soursop fruits were provided by Labu Valley, Nilai Negeri Sembilan, Malaysia. The ripe soursops were peeled, the seeds removed and the pulp obtained was homogenized using a Waring blender (Model CB6) before being frozen at −60 °C. Samples were kept at −20 °C until further use.

Methods

Crude extract preparation

Frozen soursop was blended at medium speed with 1.92 M NaCl solution using a Waring blender (Model 7011S) for 1 min. The ratio of the pulp to the extractant was 1:3; the pH was adjusted to 8.4 and maintained at that level by addition of NaOH. The slurry was centrifuged at 15 000*g* for 30 min using a refrigerated centrifuge (Beckman Model J2-21M/E) and the supernatant was collected for further purification and for assay of pectinesterase activity. All procedures were carried out at 4 °C.

Purification procedure

Ammonium sulphate fractionation Ammonium sulphate was added to the crude extract to give 40% (w/v) salt saturation. Precipitated protein was removed by centrifugation. The concentration of the ammonium sulphate in the supernatant was then increased to 90% salt saturation. The precipitate was collected by centrifugation and then dissolved in a minimum volume of

0.01 M sodium phosphate buffer, pH 7.5. After dialysis for 24 h against 0.01 M sodium phosphate buffer, pH 7.5, the insoluble residue remaining was removed by centrifugation.

Ultrafiltration Ultrafiltration was carried out using a Toyo Advantec stirred cell (molecular weight cut-off 10 000) to concentrate the enzyme solution.

Ion-exchange chromatography The dialysed enzyme solution was applied to a CM-Sephadex C-50 column that had been previously equilibrated with 0.02 M sodium phosphate buffer, pH 7.5, at a flow rate of 25 ml h^{-1}. Initially, the column was washed with the equilibrating buffer and followed by a linear gradient of up to 1 M NaCl in 0.02 M phosphate buffer, pH 7.5.

Gel filtration Fractions containing pectinesterase were pooled, concentrated and then applied to a column of Sephadex G-100. This column was eluted with 0.02 M sodium phosphate buffer (pH 7.5) with 0.2 M NaCl and 0.02% sodium azide.

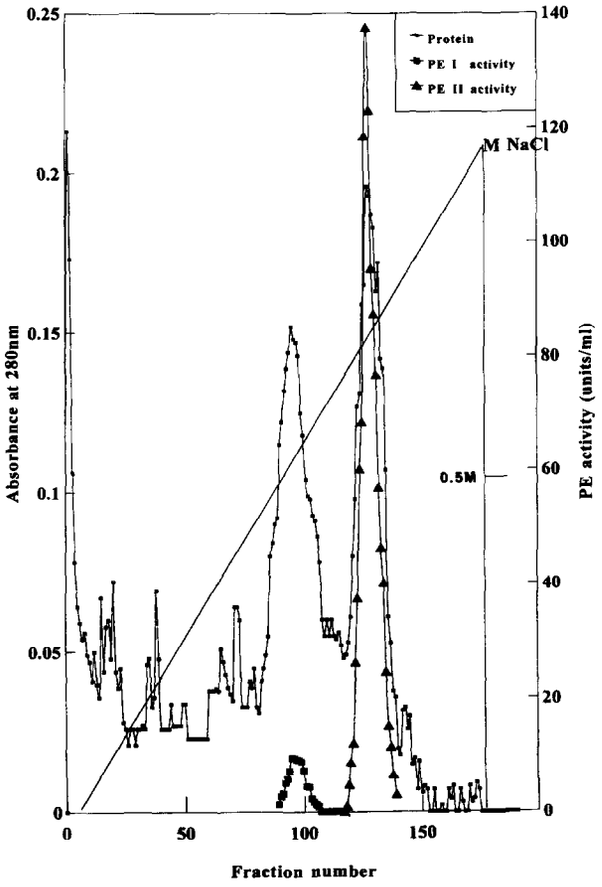

Fig. 1. CM-Sephadex C-50 chromatography. The column (2.6 cm×37 cm) was equilibrated with 20 mM sodium phosphate buffer (pH 7.5). The dialysed enzyme extract from soursop was applied to the column and washed with 500 ml of the equilibration buffer and was then eluted with a linear gradient of 0–1 M NaCl in 20 mM sodium phosphate buffer (pH 7.5) at a flow rate of 25 ml h^{-1}. The total volume of the gradient was 1000 ml. Fractions of 5 ml were collected and assayed for pectinesterase activity and absorbance at 280 nm.

Determination of pectinesterase activity

Pectinesterase activity was determined by the method of Kertesz (1955) as described by Fayyaz *et al.* (1993). Briefly, the method consists of a titrimetric measurement of the rate of carboxyl group liberation from a 1% pectin solution, at pH 7.0, containing 0.15 M NaCl at 30 °C. The released carboxyl groups were titrated with 0.02 N NaOH for 10 min using a Titralab Autotitrator Model VIT 90/ ABU 93/SAM 90 (Radiometer, Copenhagen, Denmark).

Protein determination

Protein was determined colorimetrically by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The protein content of column eluates was estimated from absorbance at 280 nm.

Determination of enzymic activity in chromatographic fractions

Fractions obtain during chromatography were assayed for pectinesterase activity using the methyl red indicator test (Versteeg *et al.* (1978). Aliquots of 10 µl of the fractions were added to small test-tubes containing 1 ml of a substrate solution composed of 1% citrus pectin,

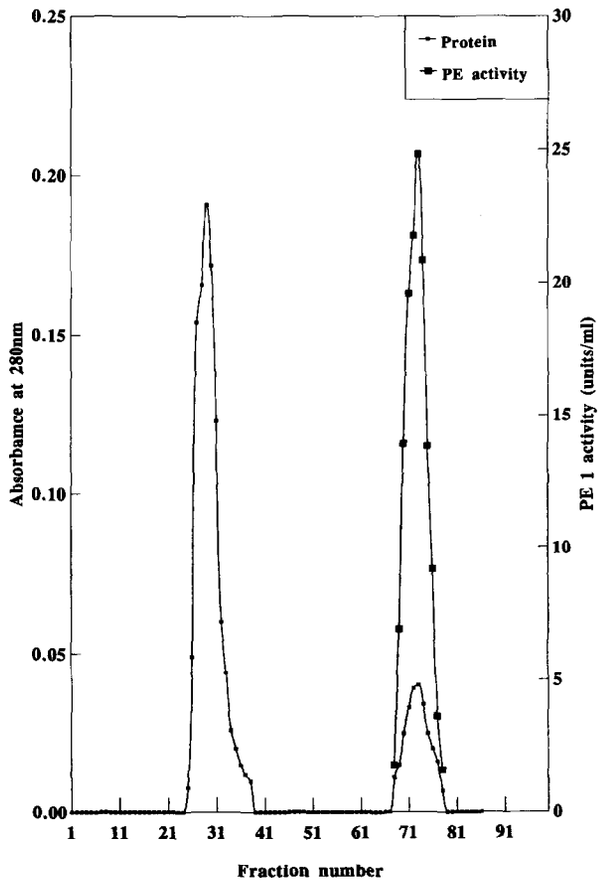

Fig. 2. Gel filtration chromatography on Sephadex G-100 of the PE I extract after CM-Sephadex chromatography. The column (2.6 cm×65 cm) was equilibrated and eluted with 20 mM sodium phosphate buffer (pH 7.5) with 0.2 M NaCl and 0.02% sodium azide at a flow rate of 2.4 ml h^{-1}.

0.15 M NaCl, 0.02% sodium azide as preservative, and a few drops of 1% ethanolic methyl red solution per 25 ml. Fractions giving a positive methyl red test (red colour) within 1 h were then assayed titrimetrically as described previously.

Molecular weight determination

The molecular weight of the purified enzymes was determined by gel filtration on Sephadex G-100. The column was calibrated for molecular weight by determining the elution volumes of standard proteins of

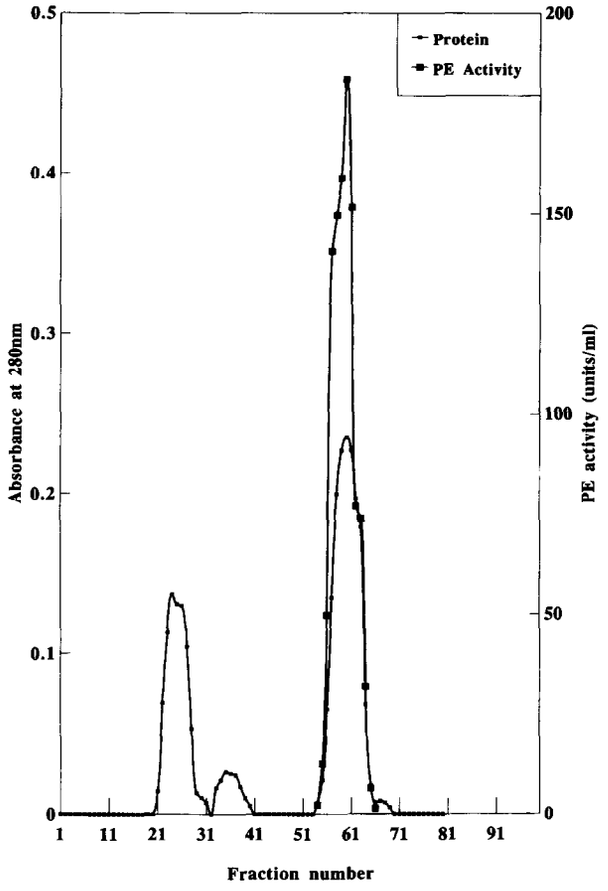

Fig. 3. Gel filtration chromatography on Sephadex G-100 of the PE II extract after CM-Sephadex chromatography. The column (2.6 cm×65 cm) was equilibrated and eluted with 20 mM sodium phosphate buffer (pH 7.5) with 0.2 M NaCl and 0.02% sodium azide at a flow rate of 3.0 ml h^{-1}.

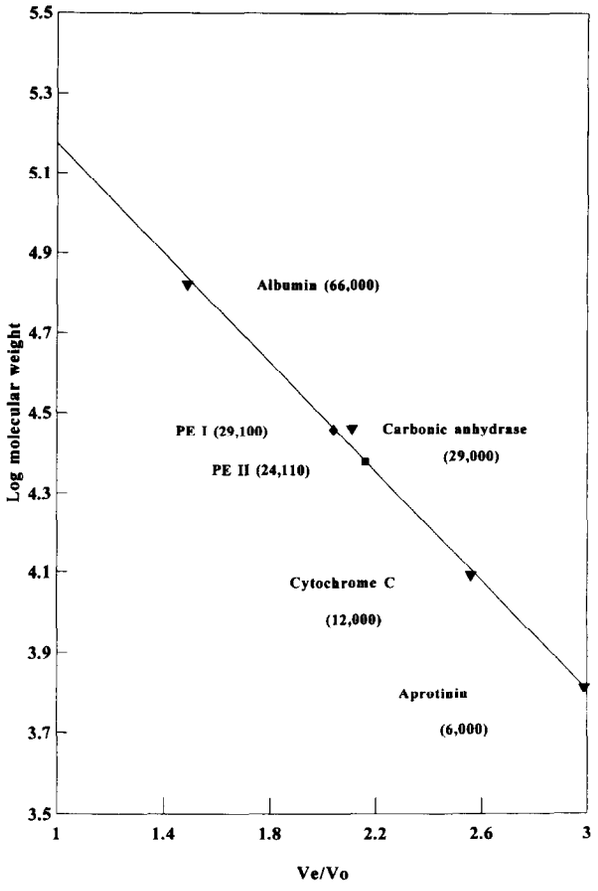

Fig. 4. Standard curve for estimating the molecular weight of soursop PE I and PE II. Elution pattern of protein (albumin, carbonic anhydrase, cytochrome *c* and aprotinin) on Sephadex G-100 column (2.6 cm×65 cm) at a flow rate 4.5 ml h^{-1}. The volume for each fraction was 5 ml.

Table 1. Purification of pectinesterase from soursop

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units mg^{-1})	Yield (%)	Fold
Crude extract, $(NH_4)_2SO_4$	1800	8680	4522	1.92	100	1
Fractionation (40–90%)	71	7526	210	35.9	86.7	19
CM-Sephadex						
Eluate						
Peak I	89	581	16.4	35.4	7	18
Peak II	109	4922	38.5	129.6	58	68
Ultrafiltration						
Peak I	13	475	11.6	41.1	6	21
Peak II	25	4786	12.4	386	55	201
Gel filtration						
Eluate						
Peak I	27	474	5.9	80	5.5	42
Peak II	40	3248	8.1	401	37	209
Ultrafiltration						
Peak I	12	331	4.1	81.7	4	43
Peak II	18	2825	6.4	439	33	229

known molecular weight. The protein standards used were bovine serum albumin (66 000), cytochrome *c* (12 000), carbonic anhydrase (28 000) and aprotinin (6500). The proteins were dissolved in sodium phosphate buffer, applied to the column and eluted at a flow rate of 4.5 ml h^{-1}; 5 ml fractions were collected. The void volume was determined with Blue Dextran 2000. The molecular weight was estimated as described by Whitaker (1963).

Gel electrophoresis

Gel eletrophoresis in denaturing conditions was performed in sodium dodecyl sulphate (SDS–PAGE) according to the method of Laemmli (1970) in the presence of 5% mercaptoethanol. Samples were heated at 100 °C for 90 s and subjected to electrophoresis with a constant current of 30 mA. Proteins were stained with Coomassie Brilliant Blue R-250 in 7% acetic acid. The molecular weight markers were albumin (66 000), ovalbumin (45 000) and trypsinogen (24 000).

pH optimum determination

The pH dependence of soursop pectinesterases was assayed titrimetrically with 0.02 M NaOH after adjusting the pH of the reaction solution to one of the pH values tested (4.5–9.0). Corrections were made to each experiment to allow for the spontaneous demethylation of pectin.

Effect of assay temperature on pectinesterase activity

The effect of assay temperature on pectinesterase activity was tested manually under standard assay conditions with varying temperatures. The temperature (10–70 °C) was controlled by means of a circulating water bath.

Determination of energy of activation (E_a)

Energy of activation (E_a) was estimated from the slope of the Arrhenius plot obtained by plotting the logarithm of the initial enzyme velocity in the standard assay against the reciprocal of the reaction temperature. The substrate concentration was many times higher than the K_m value for both pectinesterase forms.

Kinetic parameters of PE I and PE II

The effect of substrate concentration was determined by measuring activities for both purified pectinesterases in the presence of various substrate concentrations (0.03–10 mg ml^{-1}). The K_m values and the maximum

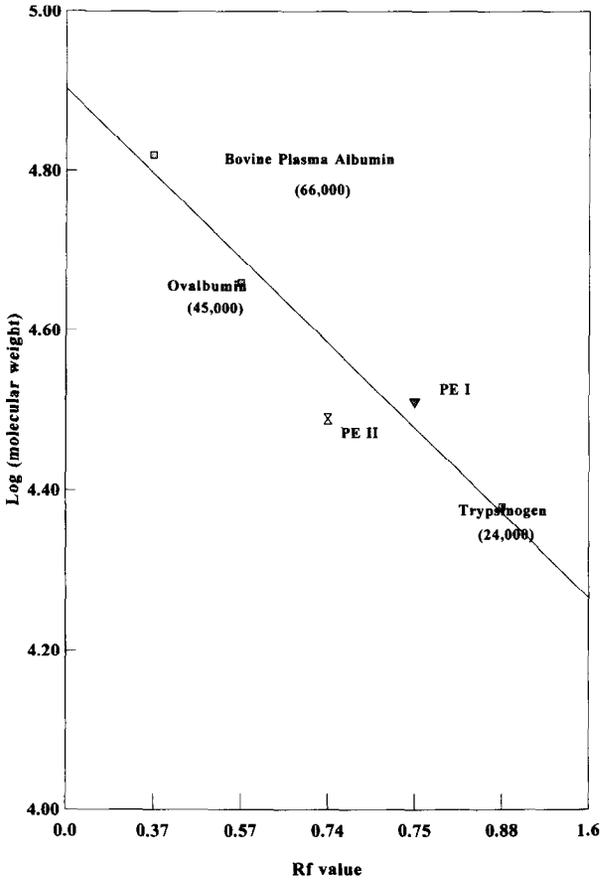

Fig. 5. Molecular weight determination of soursop pectinesterase by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE of the enzyme and standard proteins was done in a slab gel with a discontinuous buffer system. Standards: trypsinogen (24 000), ovalbumin (45 000) and bovine plasma albumin (66 000).

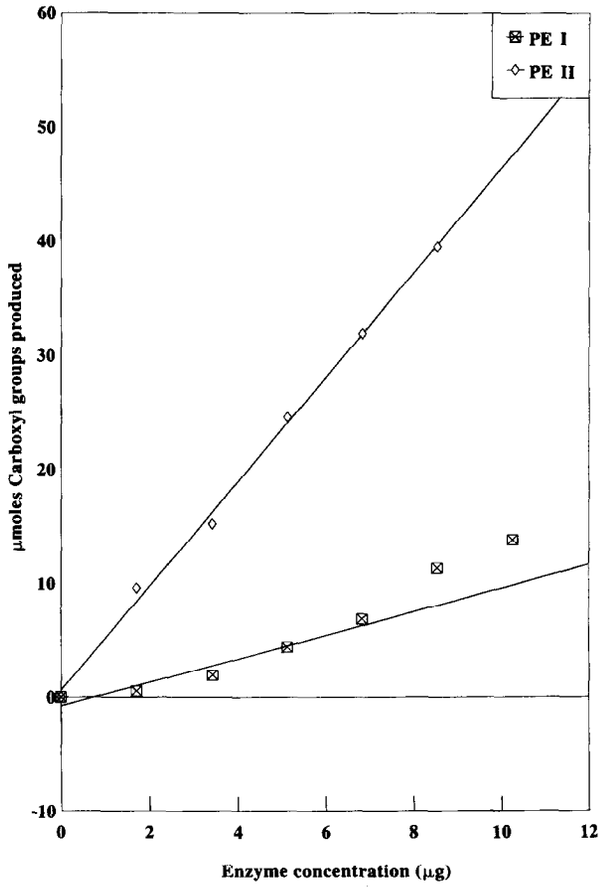

Fig. 6. Effect of enzyme concentration on purified soursop PE I and PE II activity. Measurements were carried out using 50 ml of 1% citrus pectin in 0.15 M NaCl, 0.02% sodium azide, with varying levels of enzyme.

velocities (V_{max}) were determined using the Lineweaver–Burk double reciprocal plot, in which the reciprocals of the initial velocities of the pectinesterase activity were plotted against the reciprocals of the concentration of pectin used.

RESULTS AND DISCUSSION

The 40–90% ammonium sulphate precipitate of an extract of 600 g of ripe soursop pulp was dissolved and dialysed and then subjected to chromatography on CM-Sephadex C-50. After washing, the column was eluted with a linear gradient from 0 to 1 M NaCl. Figure 1 shows the elution profiles of the proteins from the column. Two peaks of pectinesterase activity, designated as PE I and PE II, were obtained. PE I eluted first and occurred in smaller amounts than PE II. From the activities evaluated after pooling PE I and PE II separately, it can be calculated that these pools represented, respectively, about 10% and 90% of the total activity recovered after ion-exchange chromatography. The pools were concentrated by ultrafiltration and subjected to further purification by gel filtration on Sephadex G-100. Each fraction emerged in the eluate as a single peak at its specific position (Figs 2 and 3). Data published in the past few years have established that plants contain multiple forms of pectinesterase, even in the same plant tissue. Lim and Chung (1993) obtained two forms of pectinesterase, which were isoenzymes, from papaya fruits. Similarly, Bordenave and Goldberg (1993) found at least four isoforms of pectinesterase in mung bean hypocotyl cell walls.

Results of the pectinesterase purification procedure are summarized in Table 1. Approximately 4 mg (43-fold) and 6.4 mg (229-fold) of pure PE I and PE II were obtained with a yield of 4% and 33%, respectively.

The molecular weights of PE I and PE II were estimated to be 29 100 and 24 100, respectively, by gel filtration chromatography (Fig. 4). This is in good agreement with the molecular weights of tomato pectinesterases reported previously by Pressey and Avants (1972), which were also determined by column gel filtration on Sepahadex G-100 with 0.15 M NaCl (in the range of 24 300–35 500), and by Pressey and Woods (1992) on Sephadex G-75 (24 000–28 000). The two major pectinesterases each produced a single band on SDS–PAGE. Comparing their electrophoretic mobilities

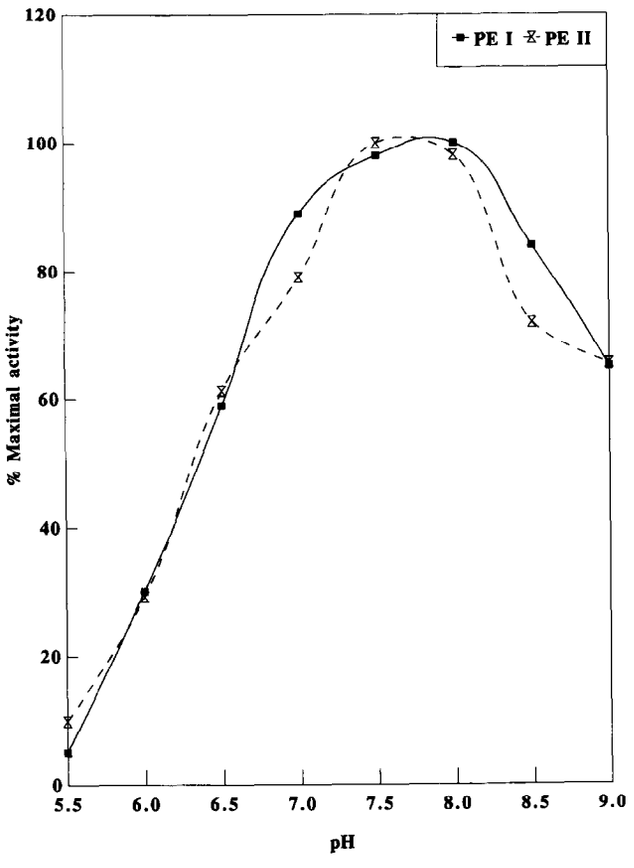

Fig. 7. The effect of pH on soursop PE I and PE II activity. The reaction mixture contained 50 ml of 1% citrus pectin in 0.15 M NaCl, 0.02% sodium azide and 10 μg of purified enzyme. The temperature of the reaction was 30 °C and the pH range 5.0–9.0.

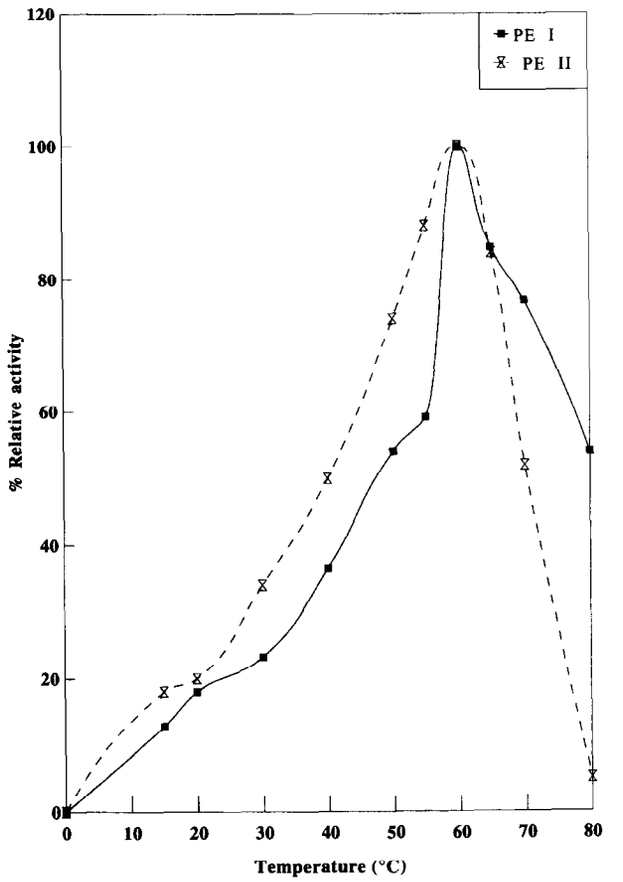

Fig. 8. Effect of assay temperature on soursop PE I and PE II activity. A 0.05 ml aliquot (1.4 unit) of PE I was added to 50 ml of preheated substrate, which contained 1% pectin in 0.15 M NaCl and 0.02% sodium azide at each temperature tested.

with those of standard proteins gave molecular weights of 31 000 for PE I and 28 000 for PE II (Fig. 5). Glover and Brady (1994) reported that the three isoforms of pectinesterase from ripe peach fruit had a molecular weight of 34 000 as determined by denaturing polyacrylamide gel electrophoresis.

Time course studies of the reaction of various concentrations of PE I and PE II showed that the reaction rate is linear with time during a period of at least 30 min. Figure 6 shows the PE I and PE II activities as a function of protein concentration. These activities increased linearly up to 8.54 μg and 10.25 μg, respectively. Puri *et al.* (1982) found that the reaction rate of pectinesterase from potato was linear with time up to 15 min with various enzyme concentrations.

The optimum pH value of the pectinesterase activity was essentially the same for both enzymes and lies in the pH range 7.5–8.0 (Fig. 7). The optimum pH found is the same as that reported by Lim and Chung (1993) and Fayyaz *et al.* (1994) for pectinesterase from papaya. Measurements of pectinesterase above pH 9.0 are not reliable because of the alkaline saponification of pectin.

The effect of reaction temperature on pectinesterase activity is shown in Fig. 8. It was observed that the pectinesterase activity increased as the temperature increased up to the optimum temperature. The optimum temperature for enzymic activity was shown to be 60 °C for both PE I and PE II. MacDonald *et al.* (1993) noted that the endocarp pectinesterase showed optimal activity at 70 °C and the peel pectinesterase was optimally active at 60 °C.

Using an Arrhenius model for activation energy (E_a), pectin de-esterification was calculated from the slope of the regression lines in Figs 9 and 10. The activation energies of PE I and PE II were 36 000 and 42 000 J mol^{-1}, respectively. This value is similar to the value reported by Javeri and Wicker (1991). The activation energy for peach pectinesterase was estimated to be 34 660 J mol^{-1}. Korner *et al.* (1980) purified two pectinesterase isozymes from shamouti orange pulp and reported activation energies of 23 442 and 24 028 J mol^{-1}, respectively.

Michaelis constants (K_m) were calculated from Lineweaver–Burk double reciprocal ($1/V$ versus 1/[S]) plots. The double reciprocal plot was linear (correlation coefficient of 0.99) over the pectin concentration range 0.03–10 mg ml^{-1}. The value for K_m is 0.52 mg ml^{-1} for PE I and 0.0843 mg ml^{-1} for PE II (Figs 11a and b). This shows that PE I had a relatively low affinity for its pectin substrate compared to PE II which was much more active at low substrate concentrations. These apparent values are within the range reported for other

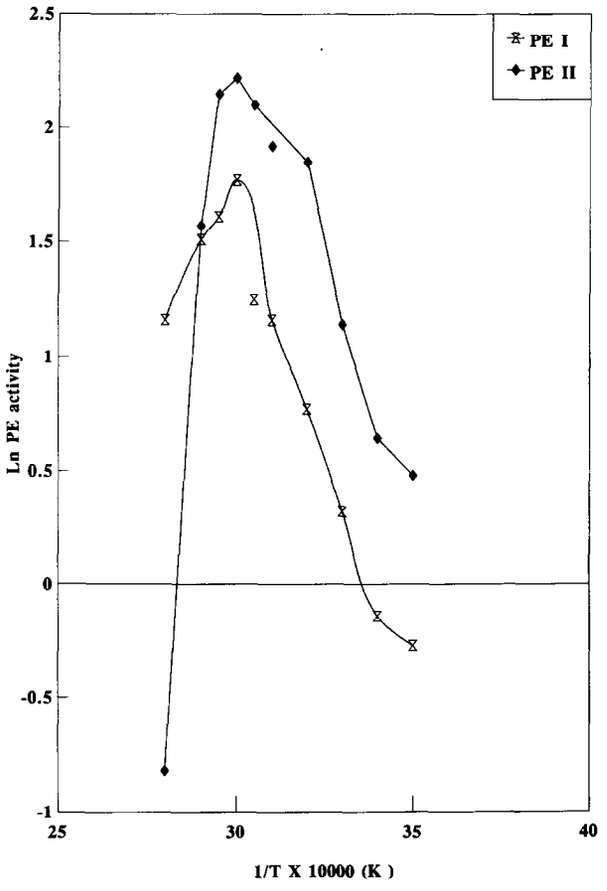

Fig. 9. Arrhenius plots of temperature effects on PE I and PE II.

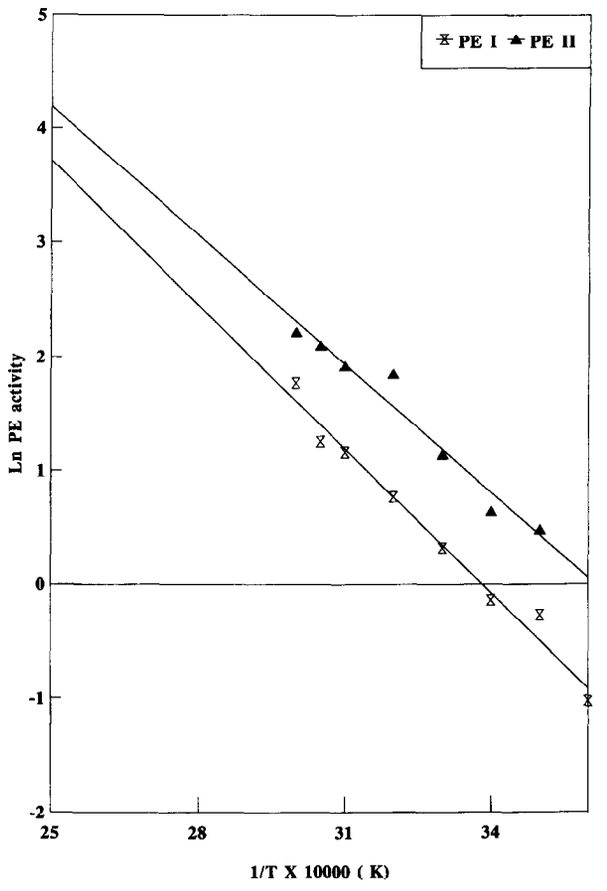

Fig. 10. Arrhenius plots of temperature effects on PE I and PE II.

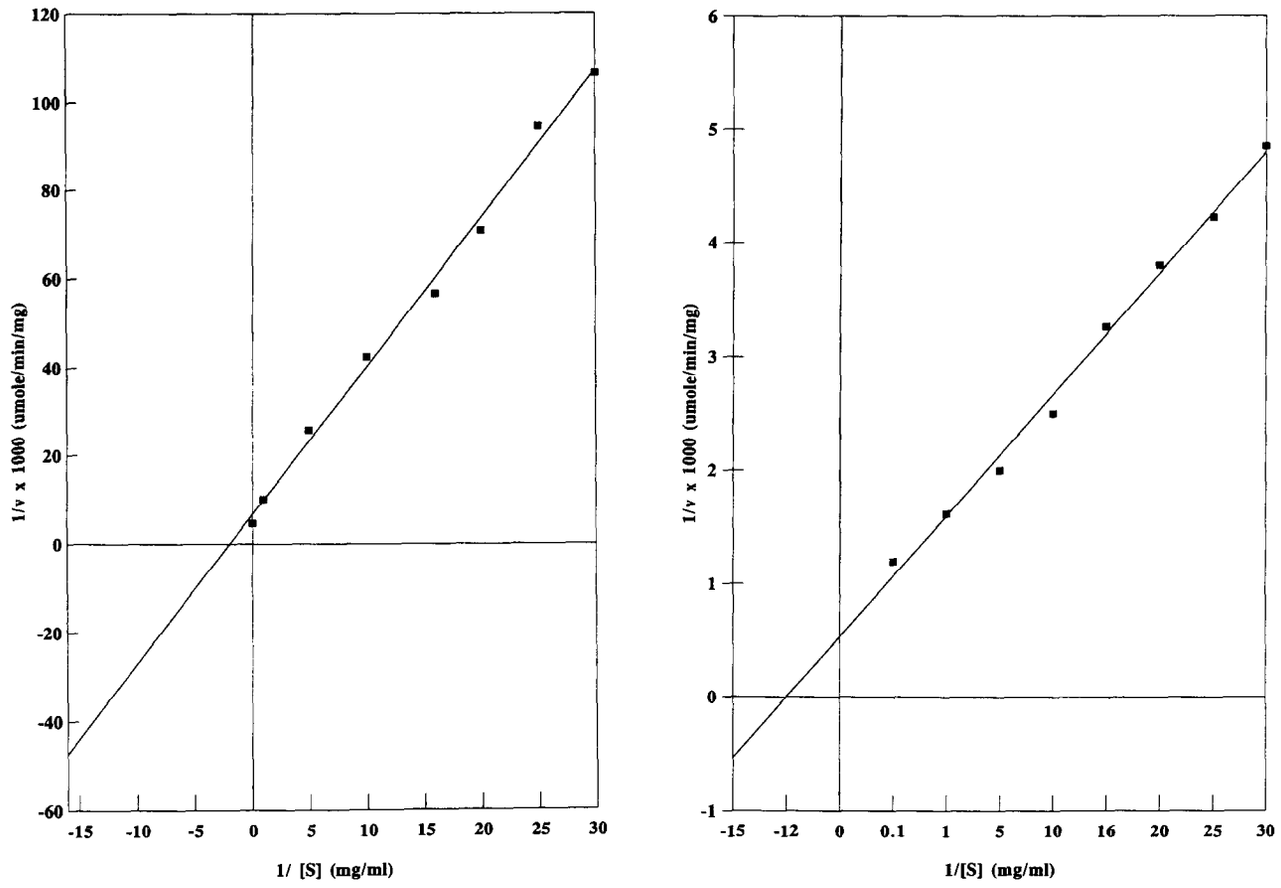

Fig. 11. Lineweaver–Burk plots of soursop PE I (a) and PE II (b) activity as a function of substrate concentration. Reaction mixtures contained varying levels of citrus pectin (0.3–10 μg ml^{-1}) in 0.15 M NaCl, 0.02% sodium azide, and 5 μg of purified enzyme. The pH of the reaction was 7.0 and the temperature was 30 °C.

pectinesterases by Rexova-Benkova and Markovic (1974) (0.71–0.166%), Lim and Chung (1993) and Lourenco and Catutani (1984) for papaya (0.04–0.24% and 0.09%), Korner *et al.* (1980) for orange (0.299–0.031 mg ml^{-1}) and Lin *et al.* (1989) for *Ficus awkeotsang* (0.75 mg ml^{-1}).

The maximal velocities (V_{max}) for PE I and PE II were calculated to be 154 and 726 μmol min^{-1} mg^{-1}, respectively. Lim and Chung (1993) reported a V_{max} for pectinmethylesterase (PME) I and PME II from papaya of 741 and 800 μmol methanol min^{-1} mg^{-1} protein, respectively, while V_{max} for awkeotsang pectinesterase was 66.67 μmol min^{-1} mg^{-1} protein (Komae *et al.*, 1990).

REFERENCES

Bordenave, M. & Goldberg, R. (1993). Purification and characterization of pectinmethylesterase from mungbean hypocotyl cell walls. *Phytochemistry*, **33**, 999–1003.

Fayyaz, A., Asbi, B. A., Ghazali, H. M., Che Man, Y. B. & Jinap, S. (1993). Pectinesterase extraction from papaya. *Food Chem.*, **49**, 373–378.

Fayyaz, A., Asbi, B. A., Ghazali, H. M., Che Man, Y. B. & Jinap, S. (1994). Purification and molecular properties of papaya pectinesterase. *Food Chem.*, **49**, 373–378.

Giovane, A., Quagliuolo, L., Servillo, L., Balestrieri, L., Laratta, B., Loiudice, R. & Castaldo, D. (1994). Purification and characterization of three isozymes of pectinmethylesterase from tomato fruit. *J. Food Biochem.*, **17**, 339–349.

Glover, H. & Brady, C. (1994). Purification of three pectinesterases from ripe peach fruit. *Phytochemistry*, **37**, 949–955.

Javeri, H. & Wicker, L. (1991). Partial purification and characterization of peach pectinesterase. *J. Food Biochem.*, **15**, 241–252.

Kertesz, Z. I. (1955). Pectic enzyme. *Methods Enzymol.*, **1**, 158.

Komae, K., Sone, Y., Kakuta, M. & Misaki, A. (1990). Purification and characterization of pectinesterase from *Ficus awkeotsang*. *Agric. Biol. Chem.*, **54**, 1469–1476.

Korner, B., Zimmermann, G. & Berk, Z. (1980). Orange pectinesterase: purification, properties and effect on cloud stability. *J. Food Sci.*, **45**, 1203–1206.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacterophage, T4. *Nature*, **227**, 680.

Lim, M. Y. & Chung, M. C. M. (1993). Isolation and characterization of pectin methylesterase from papaya. *Arch. Biochem. Biophys.*, **307**, 15–20.

Lin, T. P., Liu, C. C., Chen, S. W. & Wang, W. Y. (1989). Purification and characterization of pectinmethylesterse from *Ficus awkeotsang* makino achenes. *Plant Physiol.*, **91**, 1445.

Lourenco, E. J. & Catutani, A. T. (1984). Purification and properties of pectinesterase from papaya. *J. Food Agric.*, **35**, 1120–1127.

Lowry, H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol Chem.*, **193**, 265.

MacDonald, H. M., Evans, R. E. & Spencer, W. J. (1993). Purification and properties of the major pectinesterasae in lemon fruits (citrus lemon). *J. Sci. Food Agric.*, **62**, 163–168.

Pilnik, W. & Voragen, A. G. J. (1991). The significance of endogenous and exogenous pectic enzymes in fruit and vegetable processing. In *Food Enzymology*, ed. F. P. Fox, Elsevier Applied Science, London, pp. 7–333.

Pressey, R. & Avants, J. K. (1972). Multiple forms of pectinesterase in tomatoes. *Phytochemistry*, **11**, 3139.

Pressey, R. & Woods, F. M. (1992). Purification and properties of two pectinesterases from tomatoes. *Phytochemistry*, **31**, 1139–1142.

Puri, A., Solomon, T. & Kramer, A. (1982). Partial purification and characterization of potato pectinesterase. *Food Chem.*, **8**, 203.

Rexova-Benkova, L. & Markovic, O. (1974). Pectic enzymes and lots of padding. *Adv. Carbohydr. Chem. Biochem.*, **33**, 325–385.

Versteeg, C. (1979). Pectinesterases from the orange fruit—their purification, general characteristics and juice cloud destabilizing properties. PhD Thesis, Agricultural University, Wagenigen, The Netherlands.

Vesteeg, C. F. M., Rombout, S. & Pilnik, W. (1978). Purification and some characteristics of two pectinesterase isoenzymes from orange. *Lebens. Wissensch.u.Technol.*, **11**, 267–274.

Warrilow, A. G. S., Turner, R. J. & Jones, M. G. (1994). A novel form of pectinesterase in tomato. *Phytochemistry*, **35**(4), 863–868.

Whitaker, J. R. (1963). Determination of the molecular weights of protein by gel filtration on Sephadex. *Anal. Chem.*, **35**, 1950–1953.