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Purification and molecular properties of papaya pectinesterase

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Pectinesterase (EC 3.1.1.11) was extracted and purified from papaya (*Carica papaya* L. var. exotica). The procedure adopted for purification resulted in an approximate 250-fold purification (784 units/mg protein) with a 45% recovery of the pectinesterase activity. The enzyme was eluted in a single peak after CM-Sephadex and Sephadex G-100 chromatography. The purified enzyme had a uniform specific activity throughout the final chromatographic peak. The enzyme preparation was confirmed to be of homogeneous state by gel filtration and non-denaturing polyacrylamide gel electrophoresis and it has a molecular weight of approximately 32 000 Da.

INTRODUCTION

Pectinesterase has been purified and characterized from various plant sources such as from tomato (Lee & Macmillan, 1968), navel orange (Versteeg *et al.*, 1978), potato (Puri *et al.*, 1982), papaya (Lourenco & Catutani, 1984), apple (Castaldo *et al.*, 1989), *Ficus awkeotsang* (Komae *et al.*, 1990) and marsh white grapefruit pulp (Seymour *et al.*, 1991) and it has been found that pectinesterases from different sources have different properties such as molecular weight, specific activity etc. In fact different varieties of the same fruit have different properties (Pressey & Avants, 1972).

Due to this variation in pectinesterase properties, especially when related to processing parameters, it becomes necessary to purify and characterise the enzyme from those varieties of fruits which are commercially important. Papaya variety exotica is becoming commercially important in Malaysia and was introduced in 1987 by the Malaysian Agricultural Research and Development Institute (MARDI), Serdang. There has been much interest shown by entrepreneurs in planting the exotica on a large scale and 3240 hectares have been grown in Peninsular Malaysia in 1988 (Ahmad, 1989). A number of products can be processed from papaya and a large segment of the market of processed papaya is purée (Nordin & Adinan, 1989). Pectinesterase is one of those enzymes which has an important influence on the quality and stability of processed products. The purpose of this study was to purify and characterise pectinesterase from papaya (*Carica papaya* L. var. exotica).

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MATERIALS AND METHODS

Papaya fruits of exotica variety were supplied by the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Malaysia. Citrus pectin (P-9135, galacturonic acid content 86% and methoxyl content 9·6%), Sephadex G-100, and CM-Sephadex C-50 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and most of them were also obtained from Sigma.

Enzyme extraction

Pectinesterase was extracted according to the method developed by Fayyaz *et al.* (1993). Briefly, after thawing at 4°C, 900 g of papaya pulp (previously prepared and frozen at −20°C) were homogenised with 1800 ml of 2 M NaCl solution, pH 8·0 for 3 min in a Waring blender. After adjusting the pH to 8·0 the homogenate was incubated in a cold room at 4°C for 5 h under stirring condition. During the incubation period, the pH of the homogenate was maintained at 8·0 by adding either 2 M NaOH or 2 M HCl. The homogenate was centrifuged at 24 000 × *g* for 30 min at 4°C. The supernatant was used as the crude extract. All the procedures were carried out at 4°C.

Ammonium sulphate fractionation

The crude pectinesterase extract was brought to 30% saturation by the slow addition of solid ammonium sulphate. After allowing to stand at 4°C for 2 h, the extract was centrifuged at 24 000 × *g* for 30 min. The precipitate was discarded because it was found to

contain only a small amount of pectinesterase activity. Solid ammonium sulphate was added to the supernatant to 90% saturation and it was allowed to stand for 4 h at 4°C. The precipitate was collected by centrifugation at 24 000 × *g* for 30 min and dissolved in 0·02 M, pH 7·5 sodium phosphate buffer. The enzyme solution was dialysed for 36 h against several changes of 15 volumes of 0·02 M, pH 7·5 sodium phosphate buffer solution. The dialysed solution was clarified by centrifugation at 24 000 × *g* for 30 min and the pellet was discarded. All the procedures were carried out at 4°C.

CM-Sephadex C-50 column chromatography

The enzyme solution obtained after ammonium sulphate precipitation was concentrated by ultrafiltration using a Toyo Advantec stirred cell (molecular weight cut-off 10 000 Da). The concentrated enzyme solution was applied to a CM-Sephadex C-50 column (2·6 × 37 cm) which had been previously equilibrated with 0·02 M, pH 7·5 sodium phosphate buffer. After application of sample, the column was washed with the equilibration buffer and the enzyme was eluted by using 1000 ml linear gradient of 0–1 M NaCl in 0·02 M, pH 7·5 sodium phosphate buffer. Fractions of 5 ml were collected at a flow rate of 28 ml/h and were assayed for protein and pectinesterase activity. All the procedures were carried out at 4°C.

Sephadex G-100 chromatography

The active fractions obtained from the previous step (121–132) were combined and concentrated using the Toyo Advantec Stirred cell (molecular weight cut-off 10 000 Da). The concentrated sample was applied to a column of Sephadex G-100 (2·6 × 65 cm), equilibrated with 0·02 M, sodium phosphate buffer (pH 7·5) containing 0·2 M NaCl and 0·02 % sodium azide. The enzyme was eluted with the same buffer at a flow rate of 8 ml/h, until the absorbance at 280 nm of the effluent was negligible. Fractions of 5 ml were collected and assayed for protein and pectinesterase activity. Active enzyme fractions (36–40) were pooled, concentrated as above and stored at 4°C. The concentrated enzyme was analysed for purity and characterised. All the procedures were carried out at 4°C.

Enzyme assay

The pectinesterase activity was determined by the method of Kertesz (1955), as described by Korner *et al.* (1980). Briefly, the method consisted of a titrimetric measurement of the rate of carboxyl group liberation from 1% pectin, 0·15 M 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-enzymic 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 1 μmol of carboxyl group per min.

Determination of enzyme activity in chromatographic fractions

Pectinesterase activity in fractions obtained during chromatography was assayed using the methyl-red indicator test as described by Versteeg *et al.* (1978). Aliquots of 10–25 μl of fractions were added to small test tubes containing 1 ml of a solution (pH 7·0) composed of 1% citrus pectin, 0·15 M NaCl, 0·02% sodium azide as preserving agent 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 in the preceding section.

Protein determination

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

Analysis of purity and molecular weight determination by SDS-PAGE

The purity of the enzyme was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in slab gels using the discontinuous buffer system of Laemmli (1970). The separating and stacking gels contained a final acrylamide concentration of 10 and 4%, respectively. Staining of slab gels was done with Coommassie Brilliant Blue. The molecular weight of the pectinesterase was determined using the same electrophoresis system, using the following standard proteins: lysozyme (molecular weight 14 300 Da), β-lactoglobulin (molecular weight 18 400 Da), trypsinogen (molecular weight 24 000 Da), pepsin (molecular weight 34 700 Da), ovalbumin (molecular weight 45 000 Da) and bovine plasma albumin (molecular weight 66 000 Da).

Analysis of purity and molecular weight determination by gel filtration

The molecular weight of the native pectinesterase was also determined by gel-filtration chromatography on a Sephadex G-100 column (2·6 × 65 cm), equilibrated with 0·02 M, pH 7·5 sodium phosphate buffer containing 0·2 M NaCl and 0·02 % sodium azide. The enzyme was eluted at a flow rate of 5 ml/h and 3 ml fractions were collected. The calibration curve was prepared with aprotinin (molecular weight 6500 Da), cytochrome C (molecular weight 12 400 Da), carbonic

anhydrase (molecular weight 29 000 Da) and bovine serum albumin (molecular weight 66 000 Da). The void volume was determined with Blue Dextran 2000. The molecular weight was estimated as described by Whitaker (1963).

Non-denaturing PAGE

The non-denaturing PAGE was carried out by the modification of the methods of Bryan (1977) and Davis (1964). This procedure was adopted from the Sigma Technical Bulletin no MKR-137 (10-86). Electrophoresis was carried out using 10% polyacrylamide gel concentration.

RESULTS AND DISCUSSION

A summary of the various steps utilised to purify the pectinesterase is given in Table 1. The purification procedure carried out was repeated several times and was found to be highly reproducible. The first step of the pectinesterase purification consisted of ammonium sulphate precipitation. Both the dialysed precipitate and dialysed supernatant obtained after 0–30% and 30–90% ammonium sulphate precipitation, respectively, contained negligible quantities of pectinesterase activity. The precipitate obtained after 90% ammonium sulphate precipitation gave a 75% yield after dialysis. This value is approximately equal to the values obtained for apple pectinesterase (Castaldo *et al.*, 1989) and for *Ficus awkeotsang* pectinesterase (Lin *et al.*, 1989). However, this value is slightly higher compared to those obtained for *Ficus awkeotsang* pectinesterase (Komae *et al.*, 1990), marsh white grapefruit pulp pectinesterase (Seymour *et al.*, 1991), navel orange pectinesterase (Versteeg *et al.*, 1978) and potato pectinesterase (Puri *et al.*, 1982), the values of which are in the range of 60–67%. On the other hand, this value is lower than those of tomato pectinesterase (Lee & Macmillan, 1968), orange pectinesterase (Korner *et al.*, 1980), papaya pectinesterase (Lourenco & Catutani, 1984) and mandarin orange pectinesterase (Rillo *et al.*, 1992) which ranged from 85 to 96% yield of the enzyme activity.

The elution profile of the pectinesterase on a CM-Sephadex C-50 column is shown in Fig 1. The enzyme

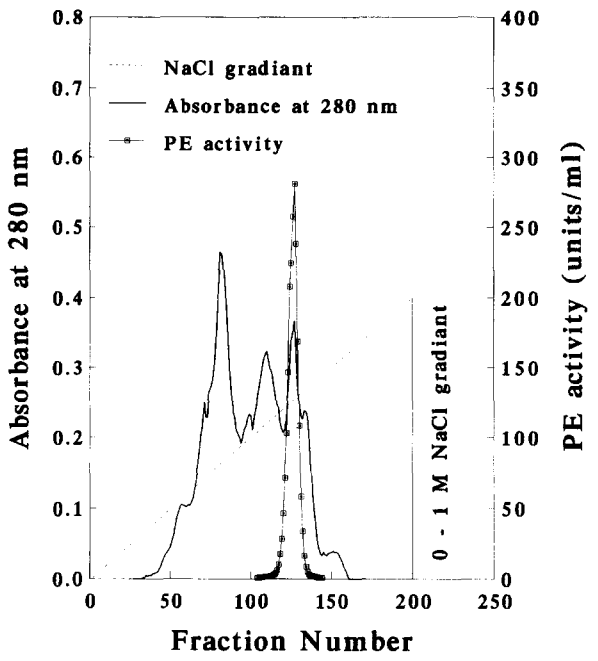

Fig. 1. Chromatography of papaya pectinesterase (PE) on CM-Sephadex C-50 column,

was found to be eluted as a single peak. This step gave a 186-fold increase in specific activity (583 units/mg protein) with a yield of 61%. A CM-Sephadex C-50 column was also used by Korner *et al.* (1980) and Seymour *et al.* (1991). Korner *et al.* (1980) have used this column (2 × 25cm) to purify orange pectinesterase by equilibrating it with 0·01 M acetate buffer (pH 5·0) and the elution of the adsorbed protein was done with two-step linear gradient increase of NaCl (0–0·2M and 0·2–0·6M). They achieved a 4·8-fold increase in specific activity (191 units/mg protein) with a yield of 58%. Likewise, Seymour *et al.* (1991) used a CM-Sephadex C-50 column to purify thermolabile pectinesterase from marsh white grapefruit pulp. They used a 2·6 × 40 cm column, equilibrated with 10 mM, pH 7·5 sodium phosphate buffer, and adsorbed enzyme was eluted with 0·2 M NaCl (pH 7·5) at a flow rate of 25 ml/h. They obtained a 58-fold increase in specific activity (403 units/mg protein) with a yield of 45%. Although the values for purification fold differ in each of these cases, it is probably due to the fact that the original activity in the crude extracts differed significantly. The activities obtained by Korner *et al.* (1980) (39·4 units/mg protein) and Seymour *et al.* (1991)

Table 1. Purification of pectinesterase from papaya fruit.

Purification steps	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Crude extract	2 585	18 043	5 764·53	3·13	1	100
Ammonium sulphate precipitate (30–90%)	157	13 532	1 082·56	12·50	4	75
Sephadex CM-C50 eluate	60	11 006	18·87	583·25	186	61
Sephadex G-100 eluate	25	8 120	10·35	784·54	250	45

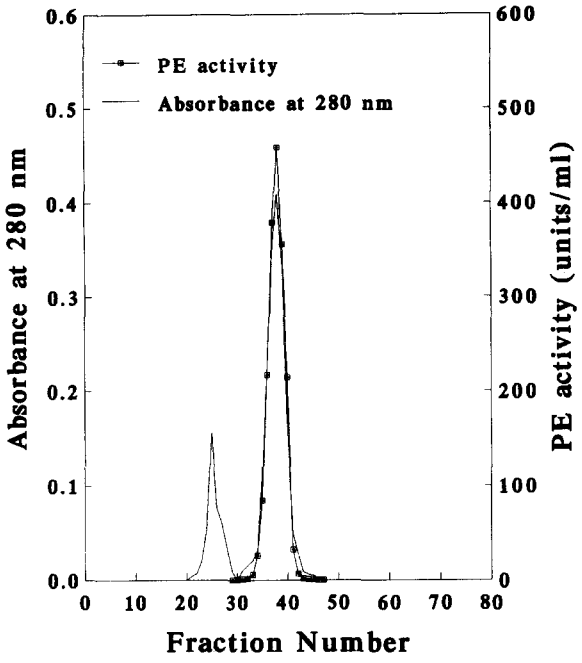

Fig. 2. Chromatography of papaya pectinesterase (PE) on Sephadex G-100 column.

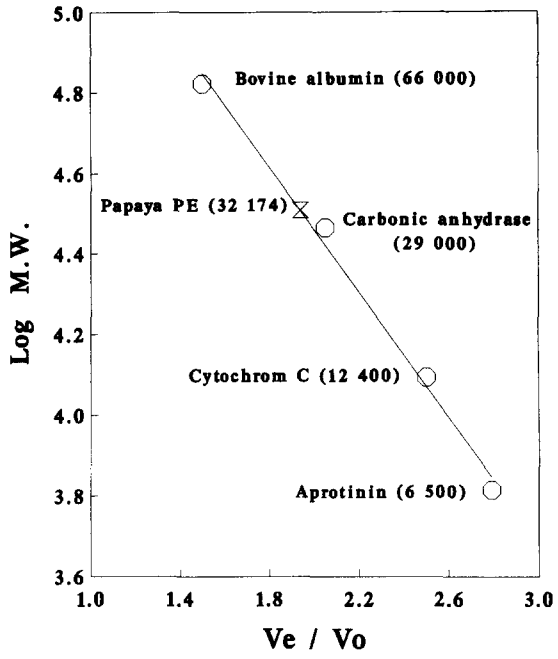

Fig. 4. Molecular weight (MW) determination of papaya pectinesterase by gel filtration on Sephadex G-100 column.

(7 units/mg protein) were higher than in the present work (3·1 units/mg protein), thus accounting for the lower eventual purification.

For further purification of this enzyme a Sephadex G-100 gel filtration column was used. The elution pattern is shown in Fig. 2. The active fractions in this step of purification (36–40) were combined and concentrated. The purified and concentrated enzyme was used for further studies.

The procedure developed in this study resulted in a 250-fold purification (784 units/mg protein) with a 45% recovery of the pectinesterase activity. The specific activity (784 units/mg protein) of the purified pectinesterase obtained in this procedure is close to the specific activity (776 units/mg protein) of a thermolabile pectinesterase purified from marsh white grapefruit pulp (Seymour *et al.*, 1991) and the specific activity (762 units/mg protein) of pectinesterase II purified from navel oranges (Versteeg *et al.*, 1978). According to Miyairi *et al.* (1975), purified apple pectinesterase also has a specific activity of 713 units/mg protein. On the other hand, the value for specific activity is very high as compared to the papaya (var. solo) pectinesterase (specific activity 35·6 units/mg) which was partially purified by Lourenco and Catutani (1984) and low as compared to the tomato (var. Heinz) pectinesterase and kiwi fruit (var. Hayward) pectinesterases I and II (specific activities 1150, 933 and 974 units/mg) purified by Lee and Macmillan (1968) and Giovane *et al.* (1990), respectively.

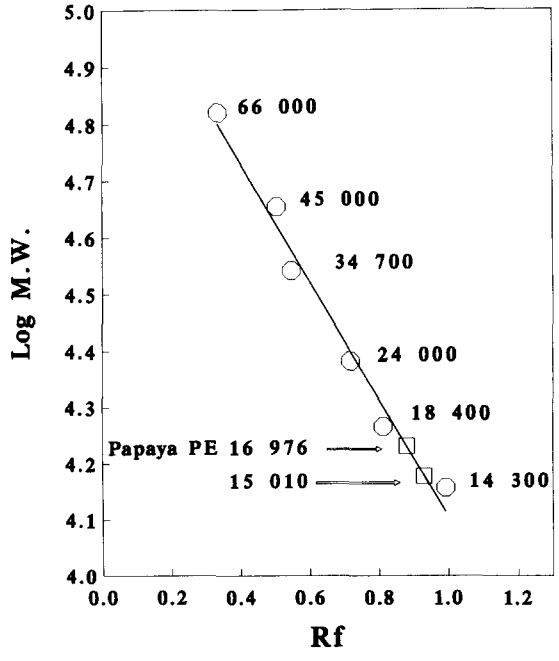

Fig. 3. Molecular weight (MW) determination of papaya pectinesterase by SDS-PAGE.

The purity of the pectinesterase in this experiment was examined by various criteria, such as, gel filtration on Sephadex G-100, non-denaturing PAGE and SDS-PAGE. The enzyme was purified to homogeneity in the last step of purification, that is by gel-filtration chromatography on Sephadex G-100 column. All of the active fractions obtained on Sephadex G-100 column have shown approximately the same specific activity and a symmetrical pattern was obtained. The purity of the native enzyme was also tested by non-denaturing PAGE and only one band of protein was observed (figure not shown). SDS-PAGE has shown that the enzyme consists of two sub-units (Fig. 3).

A molecular weight of 32 174 Da was estimated for the native enzyme by Sephadex G-100 gel filtration chromatography (Fig. 4). Non-denaturing PAGE revealed a single band of protein while SDS-PAGE showed that the native enzyme was composed of two sub-units (Fig. 3). The molecular weights of these two

Table 2. A comparison between pectinesterase purification from different varieties of papaya fruit.

Variety	Purification stage	Molecular weight (Daltons)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)	References
Solo	Partial	53 000	35·6	4·5	2·8	Lourenco & Catutani (1984)
Unknown						
PE I	Purified	21 000	N/A[a]	N/A	N/A	Lim & Chung (1989)
PE II	Purified	21 000	N/A	N/A	N/A	Lim & Chung (1989)
Exotica	Purified	32 000	784·0	250·0	45·0	This study

[a] NA, not available.

sub-units as estimated by SDS-PAGE were 15 010 and 16 976 Da, respectively. The total molecular weight of 31 986 Da of the purified enzyme obtained by SDS-PAGE is in close agreement with the molecular weight (32 174 Da) determined by gel filtration on Sephadex G-100 column. According to Delincee and Radola (1970), the apparent molecular weight for the same tomato pectinesterase could range between 24 000 and 28 000 Da depending on the method employed. Seymour *et al.* (1991) have also described the variation due to the method employed. They estimated the molecular weight of thermolabile pectinesterase as 37 300 and 35 500 Da by SDS-PAGE and gel filtration respectively while thermolabile pectinesterase has shown a molecular weight of 53 500 and 50 300 Da by SDS-PAGE and gel filtration, respectively.

The molecular weight (approximately 32 000 Da) of this pectinesterase under study lies in the range of other purified plant pectinesterases (Versteeg, 1979), but it is quite different from the other two reports available on papaya pectinesterase. Lim and Chung (1989) have described the presence of two pectinesterase isoenzymes having the same molecular weight 21 000 Da (determined by non-denaturing PAGE) in papaya fruit (variety was not mentioned). In another report, Lourenco and Catutani (1984) obtained a molecular weight of 53 000 Da (determined by gel filtration through Sephadex G-100 column) for papaya (var. solo) pectinesterase. This variation in molecular weight of pectinesterase from different varieties of the papaya fruit is supported by the results of Pressey and Avants (1972). They purified four pectinesterases from different varieties of tomatoes and found out that these pectinesterases were different in terms of their molecular weights, which ranged from 23 700 to 35 500 Da. The molecular weights of these pectinesterases were determined by gel filtration using a Sephadex G-100 column. Table 2 shows a comparison between pectinesterase purified from different varieties of the papaya fruit and it is clear that they are different in their properties.

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