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Pectin methylesterase in *Citrus bergamia* R.: purification, biochemical characterisation and sequence of the exon related to the enzyme active site

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Abstract

Three forms of pectin methylesterase (PME) were purified, from bergamot fruit (*Citrus bergamia* R.), to homogeneity by ion-exchange and affinity chromatography. The isoforms, named PME I, PME II and PME III, according their elution order on a heparin–sepharose column, were characterized for their relative molecular mass, activity kinetic parameters and thermostability. The molecular mass was estimated to be 42 kDa for the three forms, and the apparent K_m values for citrus pectin were 0.9 mg/ml for PME I and 0.5 mg/ml for PME II and PME III. The optimum pH values lie within the range 6.5–9.0, depending on salt concentration. Thermal behaviours of the three PME isoforms were studied in a temperature range from 65 °C to 80 °C with the less abundant PME I isoform showing a higher heat resistance. Moreover, the complete exon 2 sequence of PME gene was acquired (GenBank accession no. DQ458770) using a PCR-based approach on well-known *Citrus* genomic DNA present in the NCBI database.

Keywords: *Citrus bergamia*; Pectin methylesterase; Purification; Characterization; Gene sequence; Thermal stability

1. Introduction

Pectin methylesterase (pectin esterase, PME, EC 3.1.1.11) is broadly spread in plants and microorganisms. In higher plants, PME is greatly associated with the cell-wall (CW) where it is involved in pectin metabolism. PME catalyzes the de-esterification of pectin which, chemically, is methylated polygalacturonic acid to form blocks of de-esterified pectin, and releasing hydrogen ion and methanol. The actions of PME, including CW changes during fruit ripening, regulation of pollen tube growth and pathogen–host interaction, have been reported (Bosch, Cheung, & Hepler, 2005; Dorokhov et al., 1999; Micheli, 2001). In addition, PME has been intensively studied in relation to cloud stability in juices and nectars technology (Laratta et al., 1995a; Pilnik & Voragen, 1991). Texture/structure changes during processing of fruits and vegetables are closely related to changes in pectins and to the action of pectin-degrading enzymes. The presence of active PME in processed fruit and vegetables products generates demethylated pectins which can be hydrolyzed by polygalacturonase, producing shorter chains and, consequently, drastic changes of the end-product, e.g. firmness loss and decrease in viscosity (Castaldo et al., 1991, 1997; Laratta et al., 1995a). Furthermore, the PME action leads to the formation of free carboxyl groups which, in the presence of calcium ions naturally occurring in the juices, cause pectin precipitation as calcium pectate; this phenomenon is called syneresis. For these reasons, the control of PME activity, through knowledge of its dependence on parameters such as temperature, pH and saline concentration, is of

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a great importance in the food industry for optimizing the heat treatment of fruits and vegetables. Nevertheless, PME has important applications in agro-food industry. PME de-esterifying action increases the consistency of diced tomato products when immersed in calcifying solutions (Castaldo et al., 1996).

Biochemical purification and characterization in terms of properties and thermal stability of pectinesterase have been undertaken in many different fruits and plant tissues (Castaldo, Quagliuolo, Servillo, Balestrieri, & Giovane, 1989; Castro, Van Loey, Saraiva, Smout, & Hendrickx, 2004; Giovane, Quagliuolo, Castaldo, Servillo, & Balestrieri, 1990; Giovane et al., 1994; Quagliuolo et al., 2002; Rillo et al., 1992). PME in higher plants often occurs in multiple forms that frequently exhibit different molecular and kinetic properties (Cameron, Savary, Hotchkiss, & Fishman, 2005; Cameron et al., 2003; De Sio et al., 1995; Laratta et al., 1995b; Pilnik & Voragen, 1991; Pressey & Avants, 1972; Savary, Hotchkiss, & Cameron, 2002). This heterogeneity most likely reveals divergence in tissue-specific and functional specialization. In recent years, a large amount of information has been produced about the functions of PMEs in plants: they comprise abundant genomic data showing that PMEs belong to vast multigene families and the corresponding encoded enzymes are pre-pro-proteins in which the active part is preceded by an N-terminal PRO region showing a low level of identity between isoforms (Markovic & Janecek, 2004; Pelloux, Rustérucci, & Mellerowicz, 2007). Interesting, the PRO PME region shows similarities to the PME inhibitor found in kiwi fruit and Arabidopsis (Balestrieri, Castaldo, Giovane, Quagliuolo, & Servillo, 1990; Giovane, Balestrieri, Quagliuolo, Castaldo, & Servillo, 1995; Raiola et al., 2004).

Since citrus juices are of great importance in the food industry, it is therefore significant that biochemical properties, thermal stability and food application of PME from *Citrus* have been reported in detail by several authors. Versteeg, Rombouts, Spaansen, and Pilnik (1980) described the existence of three isoenzymes in orange, one of them having high molecular weight and thermal stability. Up to 12 forms of PME have been suggested to occur in citrus fruits (Pilnik & Voragen, 1991). Rillo et al. (1992) presented temperature curves for PME from mandarin fruits that retained activity up to 50 °C. Recently, in citrus fruits a putative thermostable PME of 36 kDa was purified and partially sequenced (Arias & Burns, 2002); in Valencia orange, the four isoforms reported by Cameron et al. (2003, 2005) showed different molecular weights, ranging from 34 to 42 kDa, different patterns for salt activation and pH-dependent activity and different thermal stabilities.

Although bergamot (*Citrus bergamia* Risso et Poit.), a typical citrus fruit of southern Italy, belongs to the same genus, there is no information in the literature on PME isolated from this source. Moreover, fruits of bergamot are used mostly for the extraction of its essential oil, widely employed in the cosmetic, confectionery and food industries (with an annual production of bergamot fruits corresponding to 25,000 tons) and in recent times, the utilization of its by-products has emerged in nutritional and therapeutic fields. In fact, bergamot underutilized peels and juices are still rich in sugars, fibers, pectin and flavonoids, and, therefore, they could be utilized for further technological transformations, thus, reducing ecological pollution (Gattuso et al., 2006; Statti et al., 2004; Verzera, Trozzi, Gazea, Cicciarello, & Cotroneo, 2003).

With this aim, we decided to examine PME properties in bergamot fruit since PME is one of the key enzymes involved in the control of the complex cell-wall metabolism. Three forms of bergamot PME were purified to homogeneity and afterward their biochemical properties and thermal stability were investigated. In addition, genetic analyses via PCR, based on sequence homology of known PME genes from *Citrus sinensis*, produced a partial bergamot DNA sequence corresponding to the complete exon 2 of PME gene, equivalent to the C-terminal portion of the enzyme.

2. Materials and methods

2.1. Plant and raw material

Fruits of bergamot (*C. bergamia* R.) were obtained from the experimental ground of the "Istituto Sperimentale di Agrumicoltura—C.R.A.", division of Reggio Calabria, Italy.

Citrus pectin (degree of esterification = 74%) was from Sigma–Aldrich; *S*-sepharose, HiTrap-heparin and Superdex-75 were from Amersham Bioscience (Italy); electrophoresis reagents were from Bio-Rad (Italy). All other chemicals were of analytical grade.

2.2. Extraction and purification of enzyme

Ripening fruits of bergamot were peeled manually and the endocarps (about 600 g) were homogenized with a blender; a small amount of concentrated NaOH was added to correct the pH solution to neutrality. The protein extraction was done overnight through direct addition of solid NaCl, corresponding to a 1 M concentration. All steps of the extraction and purification procedures were carried out at 4 °C, unless otherwise indicated. The suspension was centrifuged at 60,000*g* for 1 h and the pellet discarded. The supernatant (500 ml) was exhaustively dialysed against 20 mM Na acetate buffer, pH 5.5 (containing 5 mM PMSF and 5 mM 2-mercaptoethanol), and then directly subjected to chromatography on the *S*-sepharose column (2.5 × 10 cm) equilibrated in the same buffer. After washing, the bound proteins were eluted with an increasing linear gradient from 0 to 0.4 M NaCl in a total volume of 500 ml. Elution was conducted at a flow rate of 0.4 ml/min, collecting fractions of 5 ml, which were all analyzed for the absorbance at 280 nm and for the PME activity. The fractions containing PME activity were pooled and dialyzed against 20 mM Tris–HCl buffer, pH 7.5, and then concentrated by ultrafiltration on a 10 kDa cut-off Amicon filter system to

achieve a 20-fold concentrate sample. Concentrate sample (2 ml) was applied onto a HiTrap-heparin column (prepacked 5 ml) previously equilibrated in the same dialysis buffer, using an FPLC system (BioLogic DuoFlow System, Bio-Rad laboratories, USA). After washing, the column was eluted with a continuous linear gradient from 0 to 0.35 M NaCl in 20 mM Tris–HCl, pH 7.5, at a flow rate of 1 ml/min. Fractions of 1 ml each were collected and assayed for the presence of PME and their absorbance measured at 280 nm. Three peaks of PME activity, named PME I, PME II and PME III, were obtained and combined in the corresponding pools. Due to the contamination with other proteins and the low amount of PME I and PME II recovered after purification, a further chromatography step was required for these isoforms. Both pools were dialysed against 10 volumes of 20 mM Tris–HCl buffer, pH 7.5, and concentrated by ultrafiltration; then, they were purified by gel filtration using a Superdex-75 column (10/300 GL), through FPLC, equilibrated in the same buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min and 2 ml fractions were collected. This procedure was repeated until sufficient protein was enhanced for both PME I and PME II.

Protein concentration was determined by the Protein Assay Kit II (Bio-Rad), based on the Bradford method (1976), using bovine serum albumin (BSA) as standard. The purity of the PME fractions were investigated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) conducted in a Mini-Protean II cell electrophoresis unit on 12.5% homogeneous gel (Laemmli, 1970). The gel was stained with Coomassie Brillant Blue R-250 and the denatured molecular weight was estimated using LMW protein standards: albumin (67 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa).

2.3. PME assays

Routine test to measure PME activity was done by the continuous spectrophotometric assay according to the method described by Hagerman and Austin (1986). The enzymatic activity, as unit (U) of PME, was defined as the amount of enzyme needed to release 1 μmol of H^+ per min, under the assay conditions.

The titration assay for PME was used in thermal inactivation experiments and for activity studies as a function of pH and NaCl concentration (Laratta et al., 1995b). Titration was performed with 0.05 M NaOH, with 1.0% (w/v) pectin as substrate in solution containing 0.15 M NaCl in a final volume of 20 ml. The reaction was done in a thermostated cell at 25 °C, using an automatic pH-stat (Crison, mod. TT 2050). One unit (U) of enzyme is defined as the enzyme amount that releases 1 μmol of H^+, per min, from pectin in 1 ml of mixture, at pH 7.0 and at 25 °C, according to the PME activity formula:

$$(\mathrm{U/ml}) = \frac{(V - V_b) \times M \times 1000}{v \times t}$$

where V = ml of NaOH used to titrate the sample, V_b = ml of NaOH used to titrate the blank (pectic solution without sample); M = molarity of NaOH solution; v = ml of enzyme used; t = time (min) of incubation.

Control assay of pectin without PME was carried out for each pH value. The amount of acid produced, due to the spontaneous pectin demethylation by pH, was subtracted from the values obtained in the corresponding incubation mixture where PME enzyme was present.

The K_m values of isoforms were determined at various substrate concentrations (0.1–1.25 mg/ml) with 0.1 M NaCl and at pH 7.0 by double reciprocal plot via the Lineweaver–Burk equation, using a GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

2.4. Thermal inactivation of PME

100 μl of every PME, in solution containing 10 mM Tris–HCl, pH 7.0 and 100 mM NaCl were put into sealed plastic bags. Each sample was heated at a given temperature, ranging from 65 to 80 °C, in a water bath. After an appropriate incubation time, the bag was immediately chilled in liquid nitrogen and then stored at −20 °C prior to enzymatic assay. The enzyme activity was then measured by the titration method and all determinations were done in triplicate. The thermal stability was studied by plotting the residual PME activity versus time for each temperature.

2.5. Genomic DNA isolation

Young leaves from bergamot plants were used for genomic DNA extraction by a small-scale method previously described (De Masi et al., 2005). Samples were ground to powder in the presence of liquid nitrogen, using a mortar and pestle. Aliquots of 100 mg were dissolved in lysis buffer, consisting of 1.4 M NaCl, 2% (w/v) CTAB (cetyltrimethylammonium bromide), 200 mM Tris–HCl pH 8.0, 20 mM EDTA (ethylendiaminetetracetic acid), 2% (v/v) 2-mercaptoethanol, and 5 mM ascorbic acid. After that, the solution was incubated at 65 °C for 30 min and subjected to chloroform:butanol (24:1) treatment. One volume of cold isopropanol was added to the aqueous phase. The precipitate was washed in 70% ethanol, dissolved in lysis buffer and a digestion with RNase A (5 μg ml^{-1}), at 37 °C for 60 min, was undertaken to eliminate the co-extracted RNA. At last, DNA was precipitated by adding 1/10 volumes of 5 M ammonium acetate and three volume of cold ethanol. Measurement of absorbance was done to determine the purity and quantity of the DNA template. Series of DNA dilutions were calculated from the absorbance measured at 260 nm to provide 1 ng/μl solution useful in PCR reactions.

2.6. PCR and sequencing

The PCR primers were selected on the known sequence of PME exon 2 in *C. sinensis* (GenBank accession no. U82973). The forward and reverse oligodeoxyribonucleotide primers were Fex2 and Rex2 (M-Medical, Italy) of 23 and 25 nucleotides, respectively. Their sequences are as follows:

- Forward primer Fex2: 5′-TGCCACTGTCTAGCCACTGATGC-3′;
- Reverse primer Rex2: 5′-TCGAAGCCACGAATAAGACACGCTC-3′.

The reactions of amplification were carried out via PCR in 50 μl volumes containing 10 ng of DNA template from bergamot, 2 mM $MgCl_2$, 200 μM of each dNTP (Applied Biosystems, USA), 40 pmols of each primer Fex2 and Rex2, 1× Reaction Buffer (Promega, USA) and 1.25 units of Taq DNA polymerase in Storage Buffer B (Promega, USA).

The PCR mix was prepared in ice and transferred to the cool plate of a PTC-100 Programmable Thermal Controller with heated lid (M. J. Research, USA). The PCR conditions were as follows: initial DNA denaturation was for 3 min at 95 °C; then DNA amplification was done for 35 cycles, each consisting of 1 min at 95 °C, 1 min at 54 °C, 1 min at 72 °C. Finally, the reaction products were stored at 4 °C after a concluding elongation for 7 min at 72 °C. PCR products were separated by gel electrophoresis in 2% (w/v) agarose buffered with 1× TBE (89 mM Tris–borate, pH 8.4, 2 mM EDTA) at 100 V for 1.5 h. A 100 bp DNA ladder was used as standard marker of known molecular weights (Amersham Pharmacia Biotech Inc., USA). Amplified DNA fragments (amplicons) were visualized under UV transillumination by staining with ethidium bromide (0.5 μg/ml), and then digitalized by an Electrophoresis Documentation and Analysis System 120 (Kodak ds, USA). PCR assays were performed, in duplicate, to assess the consistency of the method. A negative control, without DNA template, ran with each set of amplification reactions to check contamination occurrence.

Purification of amplified DNA from TBE agarose gel was performed by using the NucleoSpin Extract II isolation kit (Macherey-Nagel, Germany). The sequence of purified DNA fragment was determined, in duplicate, by using the opposite Fex2 and Rex2 primers. The sequencing reactions were performed by the MWG Value Read service available at www.mwg-biotech.com (MWG Biotech AG, Germany).

2.7. Bioinformatics

The Fex2 and Rex2 primers were designed on the homologous sequence of *C. sinensis* by using the FastPCR programme (Kalendar, 2006). The specificity of the primers was checked by performing a sequence homology search by nucleotide–nucleotide BLAST (blastn) algorithm through all known template sequences in the public genome database of the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov.

The multiple alignment of amino acid sequences of plant PMEs, to estimate their identity percentage, was generated with the T-Coffee method (www.ch.embnet.org) developed by Notredame, Higgins, and Heringa (2000).

3. Results and discussion

3.1. Purification of bergamot PME

PME was extracted from peeled bergamot fruits by homogenization in the presence of 1 M NaCl, due to its strong ionic interaction with cell-wall, and purified through two chromatographic steps. The first step consisted of cation exchange chromatography with *S*-sepharose resin equilibrated in 20 mM Na Acetate (pH 5.5), followed by an affinity chromatography with heparin–sepharose resin equilibrated in 20 mM buffer Tris–HCl (pH 7.5). In Fig. 1, the elution profile of the heparin–sepharose column shows three peaks of PME activity named PME I, PME II and PME III, following the elution order. The PME I and PME II peaks were pooled separately, concentrated and purified to homogeneity by gel filtration chromatography using a Superdex 75 column. Finally, the three pools were concentrated and stored at –80 °C for further analyses. A typical purification procedure is summarized in Table 1; approximately 3, 10 and 60 μg of pure PME I, PME II and PME III were, respectively, obtained from 600 g of bergamot fruits. The SDS–PAGE analysis of purified PME pools is shown in Fig. 2, the three PME isoforms showed the same molecular weight of 42 kDa. The K_m for citrus pectin was determined on pure PME isoforms. The double reciprocal plot was linear (correlation coefficient of 0.99) over 0.1–1.0 mg/ml of pectin and gave a K_m

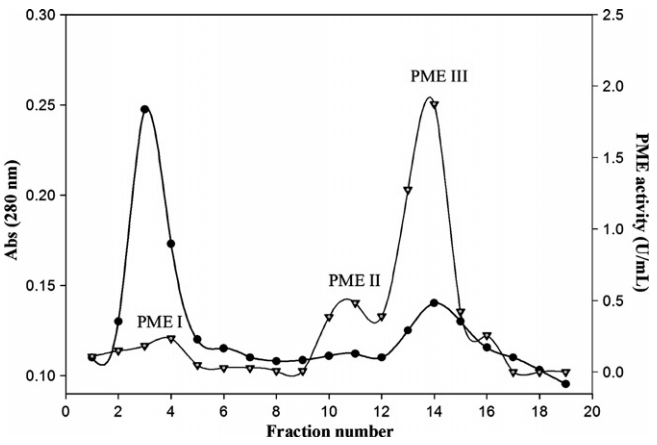

Fig. 1. Elution profile for the chromatography of bergamot PME on heparin–sepharose column. Heparin–sepharose was equilibrated in 20 mM Tris–HCl buffer (pH 7.5) as described in the text and PME activity (∇) was quantitatively measured in all fractions using a pH-sensitive dye (see Section 2). (●) Absorbance at 280 nm.

Table 1
Purification scheme of bergamot PME[a]

Purification steps	Volume (ml)	Protein (mg/ml)	Specific activity (U/mg)	Yield (%)
Homogenate	500	0.36	1.1	100
S-sepharose	150	0.2	5.75	87
PME I[b]	2	0.0015	2253	4
PME II[b]	2	0.005	2297	12
PME III[c]	2	0.03	2333	69

[a] Results are means of at least three determinations.
[b] From Superdex-75.
[c] From heparin.

value of 0.9 ± 0.07 mg/ml of pectin for PME I and 0.5 ± 0.055 mg/ml of pectin for PME II and PME III. The K_m values demonstrate that the PME I isoform had a lower affinity for pectin than had the other two isoforms that displayed the same affinity; this finding is also confirmed by the affinity chromatography experiment. In fact, PME I was poorly retained on the heparin–sepharose column, thus, showing a lower affinity for heparin that mimics the pectin structure (Giovane et al., 1990).

3.2. Characterization of bergamot PMEs

Effects of salt and optimum pH determinations were measured by the titration method, as previously described (see Section 2) taking into account the spontaneous release of hydrogen ions from pectin. In our experiments, the autohydrolysis of pectin was monitored in a pH range from 4.5 to 9.5 using the same assay protocol for PME activity but without the enzyme addition.

The activities of three purified PME isoforms were assayed at different pH values as shown in Fig. 3, PME I showed a typical pH-dependence activity with a maximum centred at pH 7.5, whereas PME II and PME III exhibited similar behaviour with two maxima corresponding to pH 7.0 and 9.0.

A different behaviour between PME I and the two isoforms, PME II and PME III, was also evidenced when the enzymes were assayed at pH 7.0 at increasing concentration of NaCl, as shown in Fig. 4. In fact a 10% increase of activity was observed at 0.1 M NaCl in all the three forms; then the activity decreased almost linearly with salt concentration increase for the PME I, while PME II and PME III showed little increase of activity when salt concentration reached 0.35 M. To further investigate this feature regarding PME II and PME III, we assayed their activity as a function of pH at two different salt concentrations,

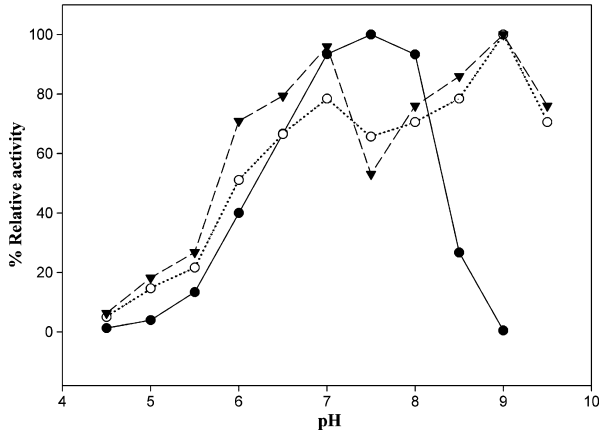

Fig. 3. PME activity as a function of pH. (●) PME I, (○) PME II, (▼) PME III.

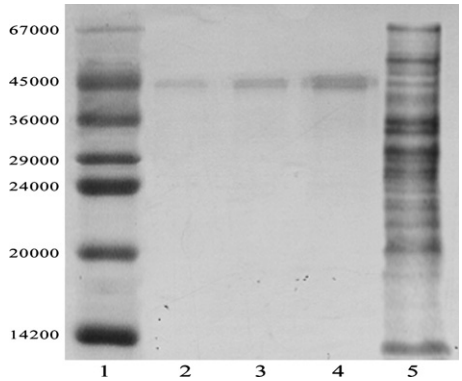

Fig. 2. SDS-PAGE analysis. Lane 1, proteins used as standards (see Section 2); lane 2, PME I from Superdex-75; lane 3, PME II from Superdex-75; lane 4, PME III from HiTrap-heparin; lane 5, homogenate of bergamot fruits.

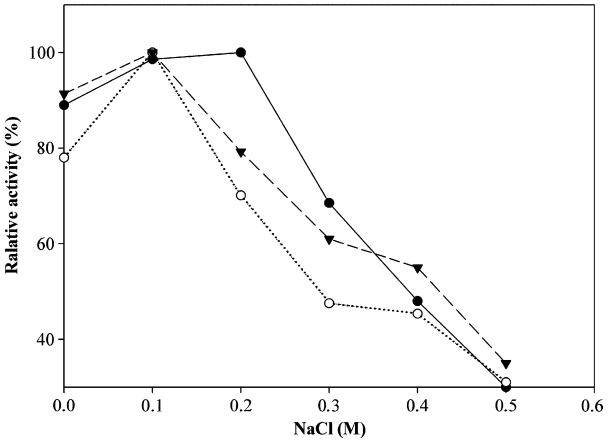

Fig. 4. PME activity as a function of NaCl: (●) PME I, (○) PME II, (▼) PME III.

as depicted in Fig. 5, where the behaviour of PME III is shown.

Two activity maxima, at pH 7.0 and pH 9.0, were found at 0.1 M NaCl whereas, at 0.35 M NaCl, only one maximum was observed, at pH 9. The activity pattern shown by bergamot PMEs at 0.1 M NaCl is also found in other PMEs (Cameron et al., 2003, 2005; Ciardiello et al., 2004; Savary et al., 2002). According to that pattern, they can be classified as salt-independent PMEs. Salts have been postulated to have an effect on PME activity by masking the carboxylic charged groups from those involved in the enzyme - substrate recognition (Cameron et al., 2003). Thus, those PMEs which are sensitive to the salt masking action are classified as salt-dependent whereas PMEs which are insensitive are classified as salt-independent. However, as far as we know, no reports are available on the activity behaviour of PME at salt concentrations higher than 0.2 M, the presence of only one activity maximum, at 0.35 M NaCl, is indicative that some modification occurs to the structures of PME II and PME III at high salt concentration. Although the salt concentration of 0.35 M is not a physiological condition, it should be noted that PME is an enzyme bound to the cell-wall via electrostatic interactions and that these interactions can modulate the PME activity, affecting its structure in a manner similar to that induced by salts. Furthermore, increasing evidence shows that plant PME could have different mechanisms of action at different pH values (Ciardiello et al., 2004). Generally, at acidic pH, a single chain mechanism (SCM) is suggested while, at alkaline pH, a multiple attack mechanism (MAM) is postulated (Ciardiello et al., 2004). Therefore salts or electrostatic interaction and pH play a fundamental role in modulating PME activity, acting not only on the number but also on the distribution of free and esterified galacturonate carboxyl groups within the pectin molecule (Willats et al., 2001). These conditions greatly influence the cell-wall firmness and, consequently, the physicochemical properties of the resulting food products. In fact, in a recent study, a dramatically different rheological behaviour has been found in lime pectins having the same methylation degree but different methyl ester distribution (Willats, Knox, & Mikkelsen, 2006).

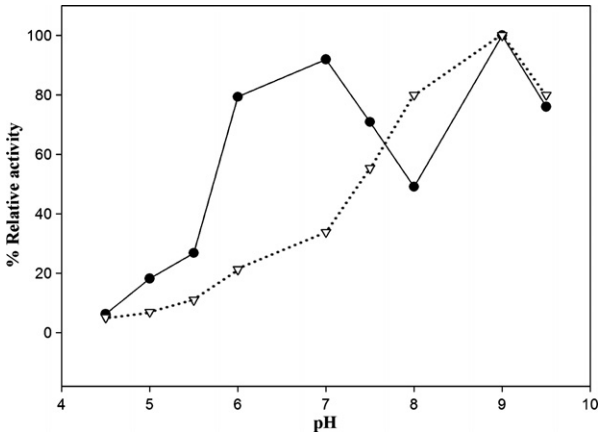

Fig. 5. Relative activity of PME III plotted versus pH in presence of (●) 0.1 M NaCl and (▽) 0.35 M NaCl.

The effect of temperature on stability of *C. bergamia* PMEs (Fig. 6) was studied by incubating each enzyme at a different temperature, ranging from 65 to 80 °C, for different times, in the range of 30–240 s in 10 mM Tris–HCl, pH 7.0, and 100 mM NaCl. PME I and PME III were quite stable at 65 °C, retaining about the 80% of their activity after 4 min of incubation whereas PME II retained 60% of activity. At 80 °C, PME I appeared to be more resistant than the other PME isoforms, retaining about 9% of its activity after 30 s where PME II and PME III, under the same conditions, retained only 1% of their activity. However, PME I, although more resistant to heat inactivation, is the less abundant form representing about 4% of total PME content in bergamot and therefore its activity gives a modest contribution to the destabilizing process in the juice.

3.3. Sequence analysis

Two groups of PME genes have been demonstrated in Valencia orange and their expression in several tissues showed that PMEs undergo post-translational modification to produce mature protein (Nairn, Lewandowski, & Burns, 1998). We have undertaken a genetic approach to determine whether, in *C. bergamia*, a specific PME isoform is encoded by a specific gene or is produced by a post-translational modification.

Within the *Citrus* genus, species are closely related; therefore, information on DNA sequences of *C. sinensis* in the GenBank database can be used for gene identification in other citrus species (Benson et al., 2002). In this way a couple of oligonucleotide primers (see Section 2) was designed on the specific DNA region of a complete PME gene sequence from *C. sinensis* (PECS-1.1, GenBank accession no. U82973). One primer (Fex2) was based on the upstream sequence of exon 2, in the intron position 3160-3182 of PECS-1.1 gene, and the second primer (Rex2) on a downstream tract of exon 2, in the 3′-UTR (untranslated region) position 4152-4176 of PECS-1.1 gene. Amplification via PCR produced four different fragments on agarose gel, as shown in Fig. 7 (lanes 1 and 2); the upper band corresponding to about 1,000 bp, indicated with the arrow, contained the exon 2 of 692 bp of the PME gene. This fragment was then isolated, sequenced and submitted to GenBank (accession no. DQ458770). The same procedure was performed for the exon 1; however, we did not find any amplification products using primers constructed on the exon 1 sequence of the PECS-1.1 gene.

Comparisons of the deduced amino acid sequence of bergamot exon 2 PME with closely related plant PMEs are presented in Fig. 8. Multiple alignments of the entire 229 amino acid residues at the C-terminal region of bergamot PME (PME-CITBE) showed significant similarity to related proteins. In particular, 100% correspondence was

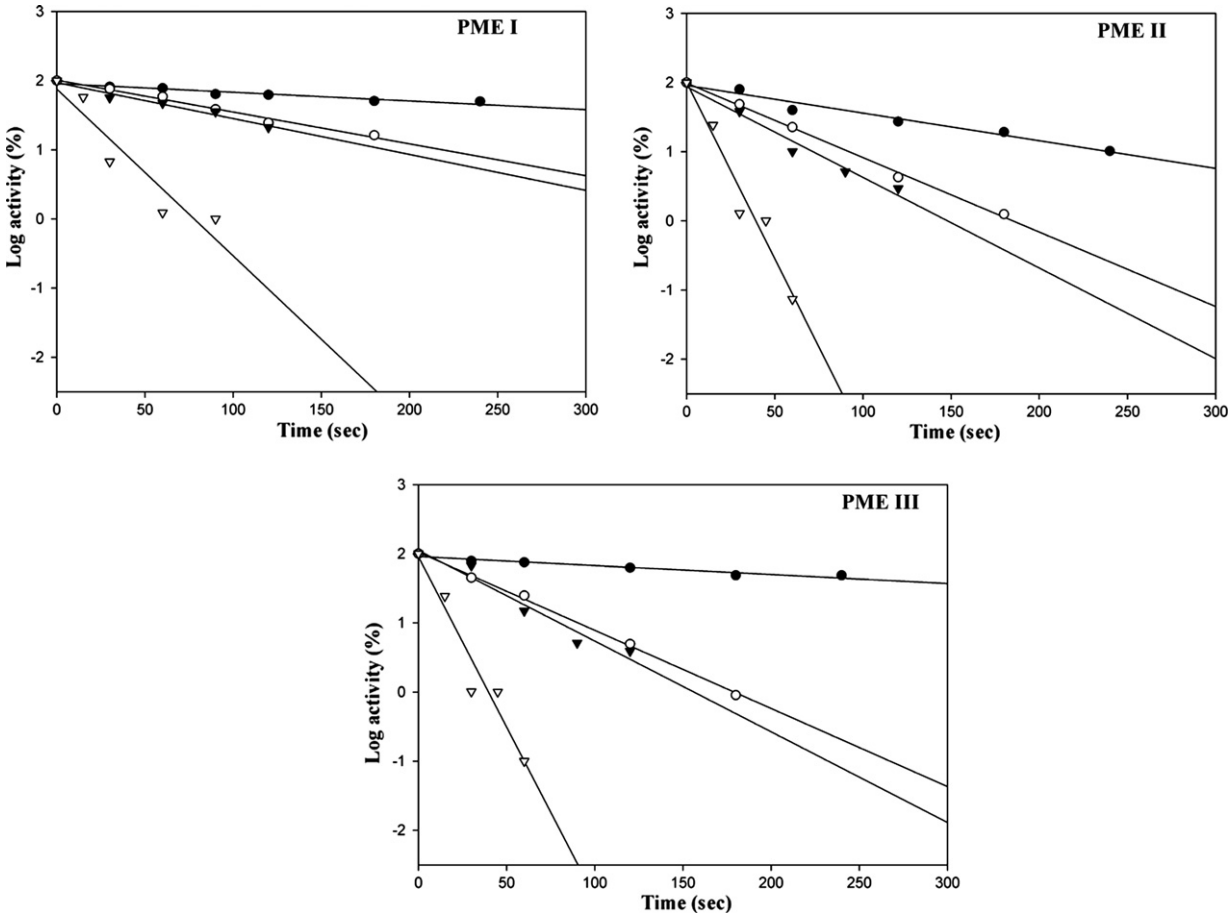

Fig. 6. Temperature curves of PME I, PME II and PME III. Residual PME activity after thermal treatments at different temperatures: (●) 65 °C, (○) 70 °C, (▼) 75 °C, (▽) 80 °C.

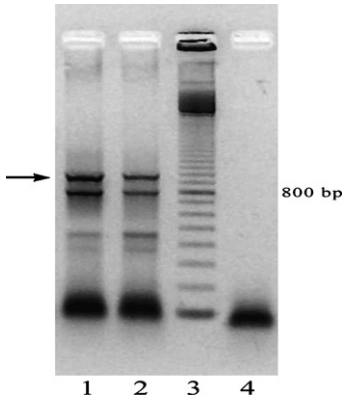

Fig. 7. PCR amplification for PME gene exon 2 on bergamot genomic DNA using the primers Fex2 and Rex2. Lane 1, PCR mix assembled with 2 mM $MgCl_2$; lane 2, PCR mix assembled with 2.5 mM $MgCl_2$; lane 3, 100 bp DNA ladder as molecular weight marker; lane 4, negative control without DNA template.

found with the sequence of PECS-1.1 from *C. sinensis* (Valencia orange, isoforms I accession no. AAB57667,) whose gene sequence was already reported as CsPME1 (Nairn et al., 1998). The correspondences with PECS-1.2 (accession no. AAB57668, group I from Valencia orange, isoforms II) and PECS-2.1 (accession no. AAB57669, group II from Valencia orange, isoforms I) (Nairn et al., 1998) were 96 and 62%, respectively. Moreover, a score of 85% was estimated with the sequenced PME DAUCA (accession no. P83218, PME from *Daucus carota*) (Johansson et al., 2002). Considering the sequence alignment, PME-CITBE is suggested to belong to the family of carbohydrate esterase CE-8 into the clade Plant 1, together with PECS-1.1 (Markovic & Janecek, 2004).

PMEs are known to be encoded by a multigene family, these genes are differentially expressed in tissues or during the ripening process in fruits. In *C. sinensis*, the exon 2, encoding for the C-terminus moiety of PMEs, is conserved while the exon 1, encoding for the N-terminus region, is variable (Nairn et al., 1998). We found that the nucleotide sequence of the entire 229 amino acid residues at the C-terminal region of bergamot PME is identical to that of CsPME1 found in *C. sinensis*. Probably the three PME isoforms in bergamot, like those of *C. sinensis*, share a common sequence at the C-terminus but have different N-termini, further investigations will be necessary to assess this hypothesis.

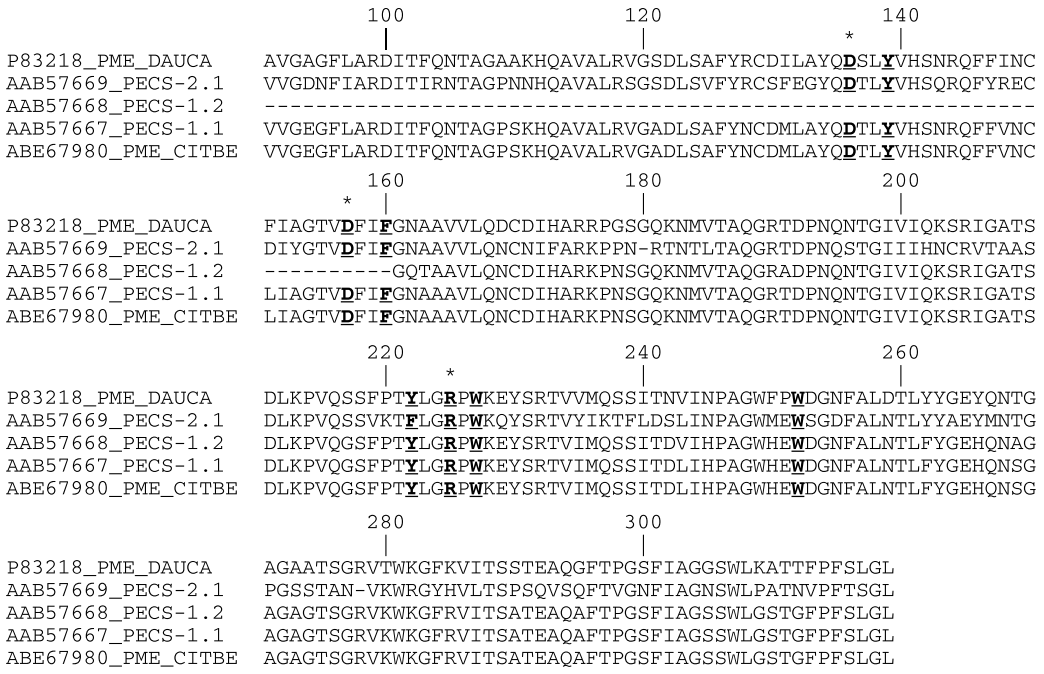

Fig. 8. Multiple alignment of CITBE deduced amino acid sequence with other related enzymes of plant PMEs. Highly conserved residues are shown in bold and underlined. The putative catalytic triad on CITBE is indicated by an asterisk (∗).

4. Conclusion

The three forms of PME isolated from bergamot fruits have a molecular weight of 42 kDa. Their optimum pH values lie within the range 6.5–9.0, depending on salt concentration. In particular they all show a salt-independent activity, but with a different behaviour between the PME I form and PME II and PME III, which were similar to each other. Differences were also found in their heat stability in a temperature range from 65 °C to 80 °C; PME I was found to be more resistant to the thermal treatment than were the other two forms. Finally, from sequencing studies, we presented evidence that PME isozymes in bergamot were consistent with those of well-characterized fruit-specific PME from *C. sinensis* (Arias & Burns, 2002). However, at this moment we do not know if the bergamot PME isoforms are the expressions of different genes or the result of different post-translational modifications.

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