Purification and Characterization of Four Pectinesterases from Sweet Cherry (*Prunus avium* L.)

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Four different pectinesterase isoforms, with different isoelectric points, have been detected and purified from sweet cherries (*Prunus avium* L.): a basic form (PE I, pI > 8.66), a neutral form (PE II, pI = 7.05), and two acidic forms (PE III, pI = 6.36); PE IV, pI = 5.24). The molecular weight (MW) of PE I was 35.4 kDa determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and 27.2 kDa determined by filtration through Sephadex G-75 SF. PEs II−IV had the same MW in SDS−PAGE (49.8 kDa) as by filtration through Sephadex G-75 SF (55.9 kDa). PEs I and IV appeared to be the most abundant forms, with K_m values of 1.03 and 74.6 μg·mL^{-1} apple pectin, respectively. The PE isoforms differ in optimum pH and thermal stability, PEs II and IV being the most thermostable. All four isoforms are activated by calcium and sodium cations and inactivated by D-galacturonic acid.

Keywords: *Sweet cherries; Prunus avium L.; Rosaceae; pectinesterase; isoenzymes; cell wall*

INTRODUCTION

Pectinesterases (PE) (EC 3.1.1.11) catalyze the hydrolysis of methyl ester groups of pectic materials. PE activity has been implicated in fruit ripening (Randhawa *et al.*, 1987; Elkashif and Huber, 1988; Wegrzyn and McRae, 1992) and primary cell wall elongation processes (Nari *et al.*, 1991; Moustacas *et al.*, 1991). During fruit maturation the pectic material in fruits is de-esterified by PE, rundering it susceptible to depolymerization by polygalacturonases. This degradation reduces intercellular adhesion, causing softening of tissues.

The enzyme has been found in all plant materials and also in fungi and bacteria pathogenic to plants (Rexová-Benková and Markovič, 1976). In fruits, two or more different isoforms of PE with different molecular weights, isoelectric points and/or other physical and chemical characteristics are usually described (Versteeg *et al.*, 1980; Castaldo *et al.*, 1989; Lin *et al.*, 1989; Seymour *et al.*, 1991a,b; Javeri and Wicker, 1991). Structural studies have shown the enzyme is a glycoprotein formed by a single low molecular weight polypeptide (Markovič and Jörnvall, 1986; Pressey and Woods, 1992; Glover and Brady, 1994) ranging between 10 kDa in banana (Markovič *et al.*, 1975) and 57 kDa in two enzymatic forms in kiwi (Giovane *et al.*, 1990). Generally their isoelectric points are between 7 and 11, although some acid forms have also been described (Markovič *et al.*, 1975; Lin *et al.*, 1989; Gaffe *et al.*, 1992).

Studies of the PE activity in sweet cherries stored in brine (Taillan *et al.*, 1992) or the frozen state (Alonso *et al.*, 1993, 1995a) suggest the existence of different isoforms of pectinesterase in cherries. The role of different PE isoforms in plant is not known. Goldberg (1984) suggested that the occurrence of PE isoenzymes could account for the changes in PE activities during plant growth.

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The control of pectinesterase activity has been a common subject of study because of its implications in the modification of the texture of fruit and vegetables (Lin *et al.*, 1989; Giovane *et al.*, 1990; Javeri and Wicker, 1991) and as a destabilizing agent of pectin materials in fruit juices and concentrates (Seymour *et al.*, 1991b; Balaban *et al.*, 1991). In sweet cherries a relationship has been established between pectinesterase activity and the firmness in fruits subjected to different thermal (Alonso *et al.*, 1993) and calcium (Alonso *et al.*, 1995a) treatments.

In this work we describe the purification and some physicochemical properties of different PE isoforms found in sweet cherries.

MATERIALS AND METHODS

Reagents. DEAE-Sephadex A-50, Sephadex G-75 SF, polybuffer 7-4, electrophoresis gels, silver staining kit, and molecular weight and isoelectric point markers were obtained from Pharmacia (Uppsala, Sweden). Apple pectin was purchased from Fluka (Buchs, Switzerland). All other reagents were of analytical grade and supplied by Merck (Darmstadt, Germany).

Enzyme Extraction. A slightly modified version of the extraction method proposed by Baldwin and Pressey (1988) was used. The fruits were collected at an optimum stage of ripeness and development (MAPA, 1987) in the Jerte Valley (Spain), where they were selected as commercial grade I with a diameter between 22 and 25 mm. All processes were carried out at 4 °C; 80 g of stoned cherries of the varieties Mollar, Lámper, Pico Colorado, Pico Negro, Ambrunés, and California was cold homogenized with 80 mL of 0.1 M NaCl, 1% Polyclar AT, and 10 mM DTT solution in a Sorvall Omni-Mixer. The pH was adjusted to 3 with 1 N HCl, and homogenates were stirred for 30 min, and then immediately centrifuged for 20 min at 12000*g*. The supernatant was collected and named *soluble enzyme extract*. The precipitate was resuspended in 100 mL of 1 M NaCl. The pH was adjusted to 6 with 1 N NaOH, and the suspension was stirred for 60 min before centrifuging at 12000*g* for 20min. The supernatant was collected and named *cell wall-bound enzyme extract*, and the precipitate was discarded. Both extracts were filtered through fiber glass filters (Whatman GF/A), and their pH was adjusted to pH 7 with 1 N NaOH.

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Solid $(NH_4)_2SO_4$ was added to both extracts up to 80% saturation, and the resulting precipitates were collected by centrifugation at $10^5 g$·min (Englard and Seifter, 1990). Pellets were resuspended in 1 mL of 1 M NaCl, and dialyzed overnight against two changes of 100 vol of 0.05 M NaCl and 20 mM Tris-HCl, pH 9.

Enzyme Purification. The purification process of the different PE isoforms was carried out from a cell wall-bound enzyme extract obtained from 1400 g of sweet cherries cv. Pico Colorado. After precipitation with ammonium sulfate, the enzyme extract was loaded on to DEAE-Sephadex A-50 (2.5 × 40 cm) columns previously equilibrated with the sample buffer. Elution was carried out with a linear gradient of 0.05−0.5 M NaCl in 20 mM Tris-HCl buffer (pH 9) at a flow rate of 13.25 mL·h^{-1}. Fractions of 10 mL were collected and assayed for protein concentration and PE activity. Fractions with PE activity of a definite peak were pooled, concentrated by ultrafiltration on Amicon PM10 membrane, and applied to a Sephadex G-75 SF column (2.6 × 65.5 cm). The column was eluted at a flow rate of 13.25 mL·h^{-1} with 0.2 M NaCl, pH 7, and fraction volumes of 4.6 mL were collected. The fractions with PE activity, containing different PE isoforms, were combined and concentrated by ultrafiltration on Amicon PM10 membrane, and the buffer was changed to 25 mM imidazole-HCl, pH 7, on a Sephadex G-25 column. The extract was applied to a 1 × 8 cm column of polybuffer exchanger PBE 7-4 equilibrated with 25 mM imidazole-HCl, pH 7, and the column was eluted with polybuffer 7-4-HCl diluted 1:8, pH 4, at a flow rate of 13.25 mL·h^{-1}. The buffer of resulting 2 mL fractions was changed to 0.2 M NaCl on Sephadex G-25 columns. The fractions were assayed for PE activity.

Native MW Determination. The molecular weight of native PE isoforms was estimated using a Sephadex G-75 SF column calibrated with a low molecular weight standards kit: ovalbumin (MW 43 000), chymotrypsinogen A (MW 25 000), and ribonuclease (MW 13 700).

Protein Determination. Total protein content was determined following the Bradford assay (1976) as modified by Sedmak and Gossberg (1977). The method includes Coomassie brilliant blue G-250 as the reactant and bovine serum albumin as the standard. The protein content in the chromatography eluent fractions was estimated by UV absorbance at 280 nm.

Enzyme Assay. During purification, PE activity was determined following the continuous spectrophotometric assay described by Hagerman and Austin (1986). The reaction mixture contained 200 μL of enzyme extract, 2.7 mL of 0.5% (w/v) apple pectin (degree of esterification 70−75%) in buffer (0.1 M NaCl and 2 mM Tris-HCl, pH 7.5, and 100 μL of 0.01% (w/v) bromothymol blue in the same buffer. The reaction was monitored at 620 nm in a recording spectrometer at a constant temperature of 25 °C. Measurements were carried out for 4.5 min at recording intervals of 0.5 min. The reaction rate ($-\Delta Abs_{620}\cdot\Delta t^{-1}$) was estimated from the tangent in the linear zone of the curves obtained. The assay was calibrated daily with a standard curve of D-galacturonic acid. The ΔAbs_{620} was linearly related to the amount of galacturonic acid added between 0 and 0.1 μmol. One unit (U) of PE activity was defined as the amount of enzyme necessary to generate 1 μmol of galacturonic acid/min in the described conditions.

Kinetic parameters were determined from the results obtained using a titrimetric assay with 0.01 N NaOH (pH 7) static at 25 °C, using a reactive mixture containing 200 μL of enzyme extract and 10 mL of 0.04−10 mg·mL^{-1} apple pectin (70−75% de) in 0.1 M NaCl, for 5 min of reaction. The activity was defined as μequiv of carboxyl groups released/min. Controls were performed with heat-denatured enzyme. The values shown are averages of three repetitions.

For the determination of optimum pH, heat stability, and effect of cations and D-galacturonic acid on the PE isoforms, PE activity was determined by production of methanol; 25 μL of PE was incubated at 25 °C with 1 mL of substrate in a screw-cap vial. This method offers high sensitivity and low enzyme consumption; however, continuous evaluation of reaction velocity is not possible. In order to ensure a constant reaction rate during enzymatic incubation, the reaction times were previously calculated from spectrophotometric PE activity assays with the purified isoforms. The reaction was stopped by heating the vial at 100 °C for 3 min. Methanol was assayed by gas chromatography following the method described by McFeeters and Armstrong (1984), using a stainless steel Carbowax (15%) 1500 (4 m × $^1/_8$ in.) column over a Chrom W, 80/100 mesh, and *n*-propanol as an internal standard. Enzyme activity was expressed in μmol of methanol produced/mL.

Electrophoretic Procedures. Electrophoretic procedures were carried out on a Phastsystem (Pharmacia) apparatus. SDS−PAGE was performed according to Laemmli (1970) on 20% polyacrylamide homogeneous gels. Phosphorylase B (MW 94 000), bovine serum albumin (MW 67 000), ovalbumin (MW 43 000), carbonic anhydrase (MW 30 000), trypsin inhibitor (MW 20 100), and lactalbumin (MW 14 100) were used as standard proteins for the estimation of protein molecular weights. Native electrophoresis was carried out on 12.5% polyacrylamide homogeneous gels maintaining a constant voltage of 400 V. Both native and SDS−PAGE were run until the bromophenol blue marker reached the anode of the gel.

For IEF, 5% polyacrylamide gels with a pH range of 3−9 were used. IEF was performed at 2000 V for 30 min, with 10 min of prefocusing prior to sample application. For the calculation of isoelectric point, 1 μL of a standard protein mixture with isoelectric points ranging between 3.50 and 8.65 was used. Gels were stained with silver nitrate according to an automatic technique developed by Pharmacia and based on the methods of Heukeshoven and Dernick (1985) and Blum *et al.* (1987). IEF and native PAGE were performed in duplicate using one gel for silver staining and the other for specific PE staining following the method described by Alonso *et al.* (1995b). Gels were analyzed using a 3CX Image Analyzer (Bio Image and Visage, Millipore Corp., Ann Arbor, MI).

RESULTS AND DISCUSSION

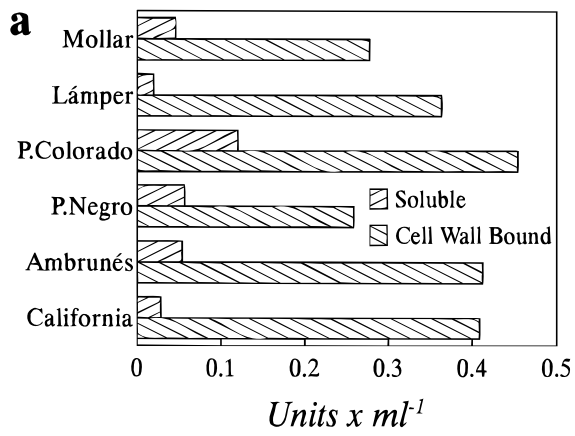
Different Isoforms of Pectinesterase. The soluble and cell wall-bound enzyme extracts of the six varieties of cherries studied showed pectinesterase activity (Figure 1a). Different PE isoforms were detected in IEF and native PAGE gels specifically stained for PE activity, and the same isoforms were found in both the soluble and the cell wall-bound enzyme extracts from the six cherry varieties. While native PAGE showed only two bands with PE activity (data not shown), IEF showed the presence of five bands with isoelectric points between 5 and 9 (Figure 1b), suggesting the presence of different PE isoforms but some with the same molecular weight.

Purification of the Different Isoforms of Pectinesterase. Among the different cherry varieties, the Pico Colorado showed a higher PE activity (Figure 1a) and was thus chosen for the isolation of the different PE isoenzymes. The soluble protein extract was not used because (i) its PE activity was lower (Figure 1a), (ii) high concentration of anthocyanins interfered with the titration of proteins, and (iii) it contained the same PE isoforms as the protein extracts from the cell wall-bound protein extracts. With 1400 g of sweet cherries cv. Pico Colorado as the starting material, the isolation process was carried out from a cell wall-bound enzyme extract containing 678 units of PE activity. A summary of the entire purification procedure is presented in Table 1.

After 80% ammonium sulfate precipitation, the enzyme extract was loaded on to a DEAE-Sephadex A-50 column equilibrated at a pH (pH 9) above the isoelectric points of the detected PE in order to retain the enzymes in the matrix. Elution in a NaCl gradient resulted in two peaks containing PE activity (Figure 2). Peak 1 eluted at a low ionic strength together with the pool of proteins not retained in the column; peak 2 was very

Table 1. Purification of Sweet Cherry Pectinesterase Isoforms

step	vol (mL)	protein (μg)	act. (units)	sp act. (units/mg)	purification (-fold)
crude extract	1494	19420	678.3	34.9	1.0
$(NH_4)_2SO_4$(80%)	12	5440	450.4	82.8	2.4
DEAE-Sephadex A-50					
peak 1	40	2340	176.8	75.5	2.2
peak 2	92	1100	284.0	258.9	7.4
Sephadex G-75 SF (peak 1)					
PE I	37	170	148.5	857.3	24.5
Sephadex G-75 SF (peak 2)					
PEs II−IV	32	30	189.7	6517.7	186.6
cromatofocusing 7-4 (peak 2)					
PE II	12	1.5	3.3	2287.7	65.5
PE III	8	3.7	5.5	1520.0	43.6
PE IV	10	6.7	44.5	6620.0	189.7



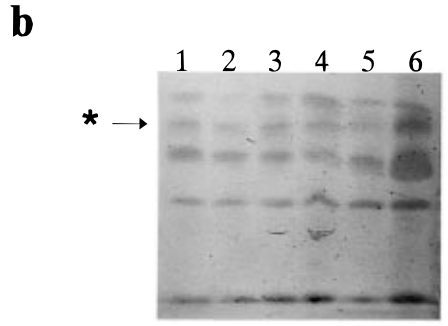


Figure 1. PE activity in six sweet cherry varieties: (a) soluble and cell wall-bound specific PE activities and (b) IEF 3−9 staining for PE with ruthenium red of Mollar (lane 1), Lámper (2), Pico colorado (3), Pico Negro (4), Ambrunés (5), and California (6) cell wall-bound enzyme extracts after ammonium sulfate precipitation. The same isoforms were detected in the soluble extracts. *→: This band with PE activity was not found after the elution by Sephadex G-75 SF gel filtration column.

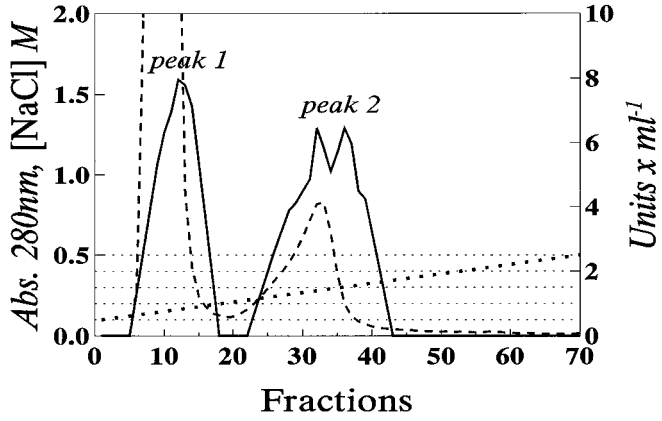


Figure 2. DEAE-Sephadex A-50 chromatography of cell wall-bound enzyme extract: (—) PE activity, (– – –) protein, and (···) gradient.

broad and eluted at an ionic strength between 0.23 and 0.35 M NaCl.

The fractions obtained from each peak were concentrated on Amicon PM10 membranes and eluted on a Sephadex G-75 SF gel filtration column. Peak 1 was resolved as a single peak with PE activity (Figure 3a), with a molecular weight estimated by gel filtration of 27.2 kDa. Peak 2 was resolved in a single peak with PE activity, with a molecular weight estimated by gel filtration of 55.9 kDa (Figure 3b). Peaks 1 and 2 were electrophoresed on IEF gels with a pH range of 3−9 (Figure 3c). Specific staining for PE activity in the IEF gels detected peak 1 as a single band, named PE I, with a pI > 8.66 (Figure 3c, lanes 2 and 3). Peak 2 on IEF 3−9 gels was found to contain three different PE isoforms named PE II (pI = 7.05), PE III (pI = 6.36), and PE IV (pI = 5.24) (Figure 3c, lanes 4 and 5). During the purification process, the fifth band with PE activity, which occurred in all six cherry varieties and had an isoelectric point somewhere between those of PEs III and IV (Figure 1b), disappeared after elution in a Sephadex G-75 SF gel filtration column. The disappearance of the fifth PE isoform could be a consequence of the change of physical elution conditions (buffer and/or pH). Thus, cherries appeared to contain four or five different molecular PE isoforms with different pIs. Peak 1 was analyzed by 20% SDS−PAGE and showed a principal band and an adjacent secondary band (Figure 3d, lane 1). However, when only the main peak 1 fractions were analyzed by 20% SDS−PAGE, PE I appeared as single homogeneous bands with an estimated molecular weight of 35.4 kDa (Figure 4b, lane 2), while peak 2 appeared as single homogeneous bands with an estimated molecular weight of 49.8 kDa (Figure 3d, lane 2). The results of SDS−PAGE do not appear to indicate the presence of subunits of the different cherry PE isoforms. The molecular weights for the four PE isoforms were within the molecular weight range of PE normally found in fruits (10−57 kDa). Gel filtration with Sephadex G-75 SF noticeably increased the specific activity (Table 1) and the purity of the PE isoforms (Figures 3c,d and 4b).

The three enzyme forms of peak 2 (PEs II−IV) were resolved by PBE 7-4 chromatofocusing (Figure 4a). The elution pHs of PE IIs and IV were close to their isoelectric points. PE III eluted at an elution pH 2 units below its pI, binding strongly to the matrix and thus requiring stronger ionic forces to become unbound. The apparent low efficiency in the recovery of PE activity from the PE II−IV forms (Table 1) after chromatofocusing on PBE 7-4 was possibly due to the difficulty in eliminating the strong buffers used in chromatofocusing, causing interferences in the measurement of PE activ-

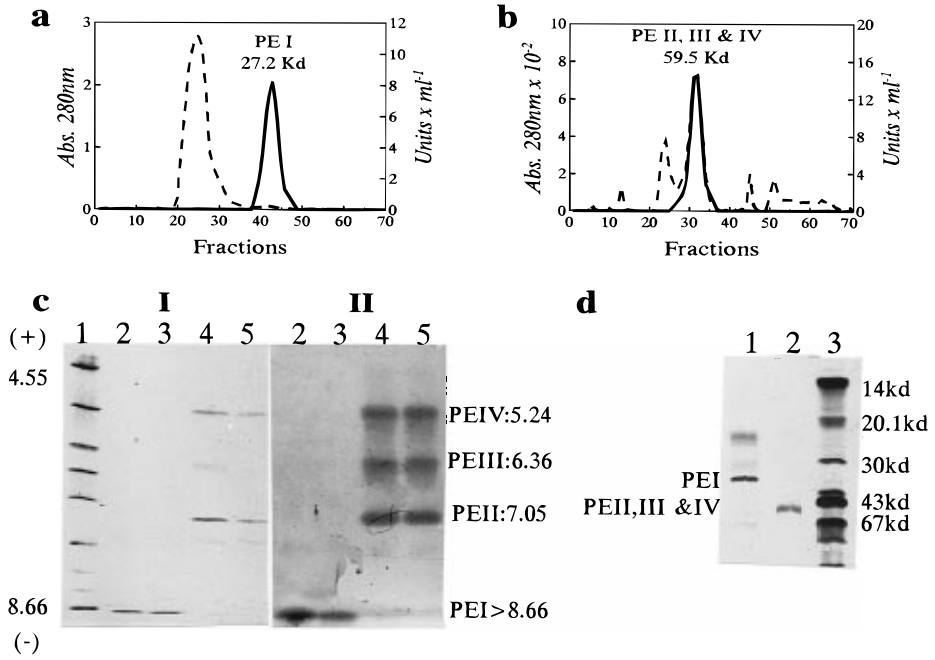


Figure 3. Chromatography of peaks 1 and 2 on Sephadex G-75 SF: elution profiles of peaks 1 (a) and 2 (b), (—) PE activity and (– – –) protein, (c) IEF 3–9 of peaks 1 (PE I) and 2 (PEs II–IV) recovered from Sephadex G-75 SF (I, silver stained; II, PE staining), (lane 1) p*I* protein standards, (lanes 2 and 3) 42 mU of PE activity from peak 1, and (lanes 4 and 5) 54 mU of PE activity from peak 2, and (d) 20% SDS–PAGE, (lane 1) 48.6 ηg of peak 1, (lane 2) 8.6 ηg of peak 2, and (lane 3) molecular weight markers.

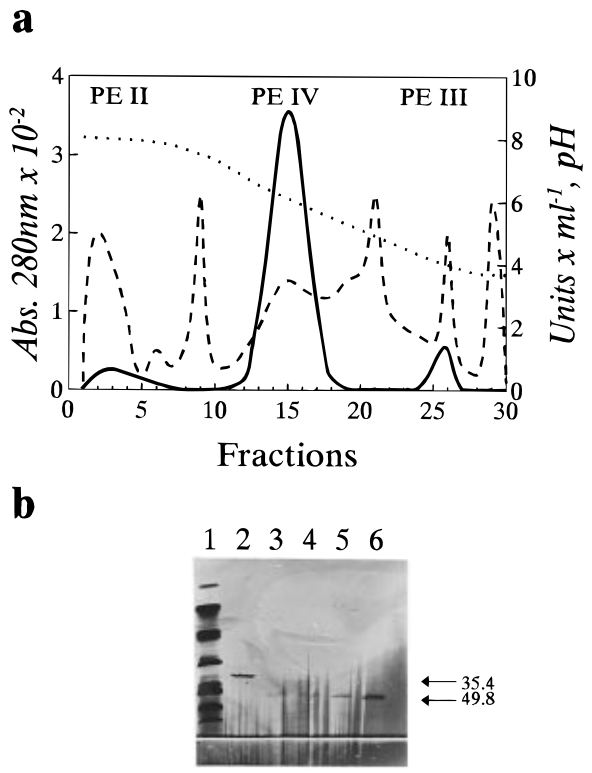


Figure 4. Separation of PEs II–IV by PBE 9-4 chromatofocusing: (a) elution profiles, (—) PE activity, (– – –) protein, and (···) pH gradient, and (b) 20% SDS–PAGE, (lane 1) molecular weight markers, (lane 2) main fractions of PE I recovered from Sephadex G-75 SF, (lane 3) 0.13 ηg of PE II, (lanes 4 and 5) 0.46 ηg of PE III, and (lane 6) 0.60 ηg of PE IV.

ity. After purification, PEs II–IV showed the same molecular weight, 49.8 kDa, estimated by 20% SDS–PAGE (Figure 4b, lanes 3–6). These results would explain why DEAE-Sephadex A-50 peak 2 was resolved into a single peak of PE activity by size exclusion chromatography on Sephadex G-75 SF (Figure 3b) and a single protein band by SDS–PAGE (Figure 3d, lane 2).

Although their overall protein content in cherries is very low (Table 1), a large fraction of the cell wall-bound protein has PE activity, and therefore cherries can be considered an abundant source of PE activity. The specific PE activity of the original extracts was very high (34.9 U·mg^{-1} of protein) in comparison with the results available from PE purification processes from other fruits: apple, 6.3 U·mg^{-1} of protein (Castaldo *et al.*, 1989); peaches, 0.72 U·mg^{-1} of protein (Javeri and Wicker, 1991); kiwi, 3.2 U·mg^{-1} of protein (Giovane *et al.*, 1990); grapefruit, 7 U·mg^{-1} of protein (Seymour *et al.*, 1991a). The specific activity attained in the PE isoforms purified, between 857 U·mg^{-1} of protein for PE I and 6620 U·mg^{-1} of protein for PE IV (Table 1), is between or above the maximum values reported from specific activities attained in PE purification processes of fruits which, with the exception of tomatoes (Lee and Macmillan, 1968) and oranges (Versteeg *et al.*, 1980), are below 1000 U·mg^{-1} of protein. However, the comparison of the results originating in different purification processes must be considered with caution due to the use of different substrates and methods for evaluating pectinesterase activity (Rexová-Benková and Markovič, 1976).

Kinetic Parameters. Of the four isoforms, PEs I and IV were the most abundant forms, accounting for approximately 90% of the PE activity of the fruit. Both isoforms differed in their affinity for apple pectin, with Michaelis–Menten values for K_m of 1.03 and 74.6 μg·mL^{-1} and V_{max} values of 1.65 and 5.41 μequiv of carboxyl groups·min^{-1}·mL^{-1}, respectively. The low K_m values, similar to those obtained for other fruit PEs, demonstrate the high affinity of these forms for their substrate.

Thermal Stability. Thermal stability of PE isoforms is shown in Figure 5. PEs I and III appeared to be the

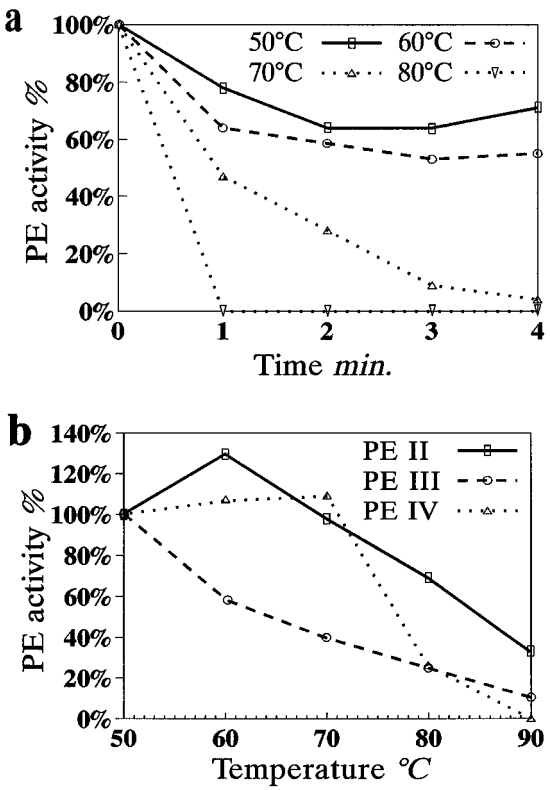


Figure 5. Heat stability of purified PE isoforms: (a) effect of temperature and application time on the activity of PE I isoform and (b) effect of temperature on the activities of PE II−IV isoforms. The isoforms were incubated at each preselected temperature for 1 min in preheated vials. PE activity was assayed with 1 mL of 1% apple pectin in 0.1 M NaCl, 0.2 M Tris-HCl, (pH 7) buffer.

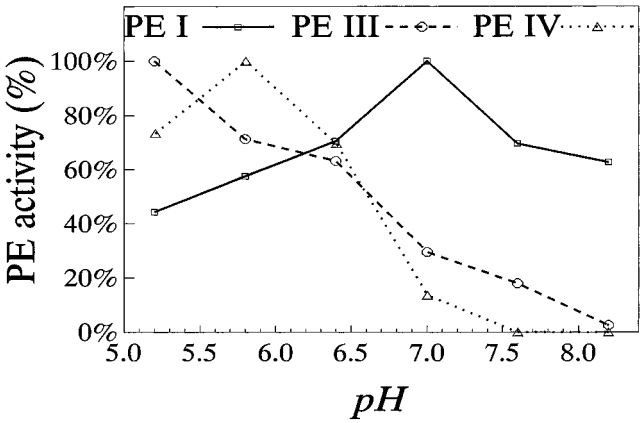


Figure 6. Effect of pH in the activity of PE I, III, and IV isoforms. PE activity was assayed with 1 mL of 1% apple pectin in 0.1 M NaCl, 0.2 M Tris-maleate, pH range 5.2−8.2.

most thermolabile isoforms, showing gradual loss of activity with increasing temperature. Both isoforms exhibited similar behavior to that of thermolabile PE isoenzymes from apple (Castaldo *et al.*, 1989) and grapefruit (Seymour *et al.*, 1991b). PEs II and IV were more thermostable, retaining 100% of their activity after heating for 1 min at 60 and 70 °C, respectively. The thermal stability of PEs II and IV can be compared to the thermostable forms of PE from orange (Versteeg *et al.*, 1980), *Ficus awkeotsang* (Lin *et al.*, 1989), and grapefruit (Seymour *et al.*, 1991b).

Optimum pH. The four isoforms displayed different behavior with respect to pH optimum (Figure 6). Only PEs I and IV showed relative maximums when enzyme activity was plotted against pH. PE I was active over the pH range 5.2−8 with an optimum pH around 7 and PE IV with a pH optimum around 5.8. No pH dependence, between 5.2 and 8, was exhibited for PE II, whereas an acidic pH optimum lower than pH 5.2 (lower limit of the range studied) was predicted for PE III. Taillan *et al.* (1992) found an optimum pH around 7 for a crude extract from sweet cherries, which is the same as the optimum pH of PE I, the majority isoform in cherries (Table 1). The pH optimum for plant PE isoforms is generally in a range from 7 to 9 (Rexová-Benková and Markovič, 1976). However plant isoforms with pH optimum in the acid range have been also described (Bordenave and Goldberg, 1993).

Table 2. Effect of NaCl and $CaCl_2$ on PE Activity of Purified PE Isoforms[a]

conctn (mM)	PE I (μmol/mL)	PE II (μmol/mL)	PE III (μmol/mL)	PE IV (μmol/mL)
		(a) NaCl		
0	1.55	0.17	0.17	1.18
10	1.97	0.52	0.89	1.77
20	2.18	1.77	0.85	1.87
40	2.74	0.77	nd	0.59
60	3.23	0.78	nd	0.74
80	3.58	0.62	nd	0.63
100	3.72	0.62	nd	0.38
200	4.21	0.51	nd	0.70
400	3.93	0.82	nd	0.78
600	3.51	0.68	nd	0.68
		(b) $CaCl_2$		
0.1	5.79	0.56	3.30	3.78
0.5	5.78	0.54	3.02	3.74
1	7.45	0.90	6.51	4.79
5	9.61	0.47	6.31	5.94
10	10.19	0.63	6.38	5.47
50	8.34			
100	7.42			

[a] The PE was assayed with 1 mL of 1% apple pectin in 0.2 M Tris-HCl, (pH 7) buffer and NaCl concentration of 0−600 mM or $CaCl_2$ concentration of 0−100 mM. nd: value not detected.

Effect of Cations. The effects of Na^+ and Ca^{2+} on PE isoform activities are shown in Table 2. The optimum concentration of Na^+ for PE I was found around 200 mM, with 75% of optimun activity being retained at 600 mM NaCl. Lower optimum Na^+ concentrations were found for PEs II−IV, between 10 and 20 mM NaCl, showing inhibition at NaCl concentrations above 40 mM (Table 2a). The optimum concentration of Na^+ for the four isoforms was in the range of those found for other PE from plants (Rexová-Benková and Markovič, 1976). The four isoforms were activated by $CaCl_2$ concentration between 0.5 and 1 mM, and 70% (PEs I and II), 98% (PE III), and 92% (PE IV) of maximal activity was retained at higher calcium concentrations (Table 2b).

Inhibition by D-Galacturonic Acid. The four isoforms were inhibited by D-galacturonic acid (Table 3). In PEs I and IV inhibition started at concentrations over 10 mM D-galacturonic acid, remaining 24% and 18%, respectively, of the initial activity at the highest D-galacturonic acid concentration, 30 mM, used. PEs II and III were more strongly inhibited, losing 98% and 92% PE activity, respectively at 30 mM D-galacturonic acid concentration (Table 3).

Pectinesterase activity outstripped by far the net changes in methylation occurring during ripening of the fruit (Rhodes, 1980), but the factors controlling this activity are not full understood. Pectinesterase appears to be involved in cell wall extension processes, and its activity may then be controled by metallic ions and the

Table 3. Effect of D-Galacturonic Acid on the PE Isoform Activities[a]

D-galacturonic acid (mM)	PE I (μmol/mL)	PE II (μmol/mL)	PE III (μmol/mL)	PE IV (μmol/mL)
0	0.78	0.93	0.65	1.52
10	0.89	0.53	0.42	1.38
20	0.31	0.16	0.19	0.55
30	0.19	0.02	0.05	0.27

[a] The PE activities were assayed with 1 mL of 0.50% apple pectin in 0.1 M NaCl, 0.2 M Tris-HCl (pH 7) at different concentrations of the inhibitor.

pH of the medium (Ricard and Noat, 1986; Moustacas *et al.*, 1991). Activation of PE by metallic ions appears to be due mainly to interaction of the ions with the substrate rather than with the enzyme (Nari *et al.*, 1991). The metallic ions would release the enzyme molecules trapped in the blocks of free carboxyl groups, enabling them to reactivate. In this way, a number of authors have reported inhibition of PE activity by polygalacturonic acid of a competitive type (Manjon *et al.*, 1992; Pitkaenen *et al.*, 1992; Fayyaz *et al.*, 1995). However, the principal texture changes during ripening of cherries coincide with sharp increases in polygalacturonase (PG) and PE activity (Barrett and Gonzalez, 1994) decreasing the number of blocks of free carboxyl groups. PE reduced the degree of esterification of pectins, rendering them susceptible to degradation by PG. Our own results suggested that D-galacturonic acid resulting from degradation of polygalaturonic acid by PG could be one of the factors controlling PE activity during senescence of the fruit, halting the reaction and preventing excessive degradation of pectic material in the ripe fruit. Inhibition by D-galacturonic acid has also been reported in PE isoforms of persimmon fruit (Alonso *et al.*, 1995c), carrot (unpublished data), and *Erwinia chrysanthemy* (Pitkaenen *et al.*, 1992).

In addition to the physiological ripening process in cherries (Barrett and Gonzalez, 1994), PE activity has been found to be involved in changes in the pectic materials responsible for maintaining the firmness of fruit during technological processing (Taillan *et al.*, 1992; Alonso *et al.*, 1995a). The present results provide an explanation for previous findings and a basis for proposing guidelines on mechanisms to regulate pectinesterase activity in sweet cherries *in vivo* with a view to preserving the texture of the fruits during technological processes. Thus, the information gained on heat stability of PEs II and IV could be used in the application of low-temperature heat treatments in order to modify the composition of the cell wall pectic material. According to the theory of Bartolome and Hoff (1972), heating at 60 °C causes loss of membrane selective permeability, giving rise to diffusion of cations to the cell wall. The increased presence of cations in the cell wall would activate the thermostable PE isoforms, augmenting de-esterification of pectins and facilitating the formation of divalent bridges between residues of galacturonic acid belonging to adjacent pectic chains. The divalent ion−pectin complex thus formed acts as an intercellular cement to give firmness to tissues. Thermal pretreatments at 70 °C prevent freezing-induced loss of cherry fruit firmness (Alonso *et al.*, 1993), increase PE activity and the pectin fraction soluble in EDTA, and reduce the degree of pectin esterification (Alonso, 1993).

Similarly, activation of the four purified PE isoforms by Ca^{2+} can be used to prevent loss of fruit firmness. Postharvest treatments with calcium have been carried out on cherries to attenuate the damage produced during mechanical harvesting and manipulation or to increase their chilled storage life (Lidster *et al.*, 1979; Facteau *et al.*, 1987). The levels of calcium in cherries have been associated with their firmness (Lidster *et al.*, 1979; Facteau *et al.*, 1987; Alonso *et al.*, 1995a), the formation of calcium pectate (van Buren, 1967; Glenn and Poovaiah, 1989; Alonso *et al.*, 1995a), and PE activity (Alonso *et al.*, 1995a).

The presence of different PE isoforms in fruits may be due to their supposed involvement in a number of physiological processes: fruit softening, abscission, cell wall extension, etc. (Gaffe *et al.*, 1994). An understanding of the purification process and characterization of the different PE isoforms will provide a basis for further studies on their physiological role. Nonetheless, the low protein levels in PE isoforms of cherries present difficulties for, and raise the cost of, sequencing of amino acids and isolation of antibodies for enzyme immunolocation studies. In view of their low protein concentration, the next step in examination of the different PE isoforms of cherries ought perhaps to be overexpression of cherry PE isoforms.

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LITERATURE CITED

Alonso, J. Estudio de la influencia de distintos tratamientos en la textura de cerezas (*Prunus avium* L.) congeladas. Función de la pectinesterasa. Ph.D. Dissertation, Universidad Complutense de Madrid, Madrid, Spain, 1993.

Alonso, J.; Canet, W.; Rodríguez, M. T. Effect of various thermal pre-treatment on the texture of frozen cherries (*Prunus avium* L.). Related enzyme activities. *Z. Lebensm.-Unters. Forsch.* **1993**, *196*, 214−218.

Alonso, J.; Rodríguez, M. T.; Canet, W. Effect of calcium pretreatments in the texture of frozen cherries. role of pectinesterase in the changes in the pectic materials. *J. Agric. Food Chem.* **1995a**, *43*, 1011−1016.

Alonso, J.; Rodríguez, M. T.; Canet, W. Detection of pectinesterase in polyacrylamide gels. *Electrophoresis* **1995b**, *16*, 39−42.

Alonso, J.; Howell, N.; Canet, W. Purification and characterization of persimmon fruit pectinesterases. *7th Cell Wall Meeting*; Universidad de Santiago de Compostela: Santiago de Compostela, Spain, 1995c.

Balaban, M. O.; Arreola, A. G.; Marshall, M.; Peplow, A.; Wei, C. I.; Cornell, J. Inactivation of pectinesterase in orange juice by supercritical carbon dioxide. *J. Food Sci.* **1991**, *56*, 743−746, 750.

Baldwin, E. A.; Pressey, R. Tomato polygalacturonase elicits ethylene. Production in tomato fruit. *J. Am. Soc. Hortic. Sci.* **1988**, *113*, 92−95.

Barrett, D. M.; Gonzalez, C. Activity of softening enzymes during cherry maturation. *J. Food Sci.* **1994**, *59*, 574−577.

Bartolome, L. G.; Hoff, J. E. Firming of potatoes. Biochemical effects of preheating. *J. Agric. Food Chem.* **1972**, *20*, 266−270.

Blum, H.; Beier, H.; Gross, H. J. Improved silver staining of plant proteins, RNA, and DNA in polyacrylamide gels. *Electrophoresis* **1987**, *8*, 93−99.

Bordenave, M.; Goldberg, R. Purification and characterization of pectin methylesterases from mung bean hypocoltyl cell wall. *Phytochemistry* **1993**, *33*, 999−1003.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Castaldo, D.; Quagliuolo, L.; Servillo, L.; Ballestrieri, C.; Giovane, A. Isolation and characterization of pectin methylesterase from apple fruit. *J. Food Sci.* **1989,** *54*, 653−673.

Elkashif, M. E.; Huber, D. J. Enzymic hydrolysis of placental cell wall pectins and cell separation in watermelon (*Citrullus lanatus*) fruit exposed to ethylene. *Physiol. Plant* **1988,** *73*, 432−439.

Englard, S.; Seifter, S. Precipitation techniques. In *Methods in Enzymology*; Deutscher, M. P., Ed.; Academic Press: San Diego, CA (EEUU), 1990; Vol. 182.

Facteau, T. J.; Rowe, K. E.; Chestnut, N. E. Response of Bing and Lambert sweet cherry fruit to preharvest calcium chloride applications. *HortScience* **1987**, *22*, 271−273.

Fayyaz, A.; Asbi, B. A.; Ghazali, H. M.; Man, Y. B. C.; Jinap, S. Kinetics of papaya pectinesterase. *Food Chem.* **1995**, *53*, 129−135.

Gaffe, J.; Morvan, C.; Jauneau, A.; Demarty, M. Partial purification of flax cell wall pectin methylesterase. *Phytochemistry* **1992**, *31*, 761−765.

Gaffe, J.; Tieman, D. M.; Handa, A. K. Pectin methylesterase isoforms in tomato (*Lycopersicon esculentum*) tissues. Effects of expression of a pectin methylesterase antisense gene. *Plant Physiol.* **1994**, *105*, 199−203.

Giovane, A.; Quagliuolo, D.; Servillo, L.; Balestrieri, C. Pectin methyl esterase from *Actinidia chinensis* fruits. *Phytochemistry* **1990**, *29*, 2821−2823.

Glenn, G. M.; Poovaiah, B. W. Cuticular properties and postharvest calcium applications influence cracking of sweet cherries. *J. Am. Soc. Hortic. Sci.* **1989**, *114*, 781−788.

Glover, H.; Brady, C. Purification of three pectin esterases from ripe peach fruit. *Phytochemistry* **1994**, *37*, 949−955.

Goldberg, R. Changes in the properties of cell wall pectin methylesterase along the *Vigna radiata* hypocotyl. *Physiol. Plant.* **1984**, *61*, 58−63.

Hagerman, A. E.; Austin, P. J. Continuous spectrophotometric assay for plant pectin methyl esterase. *J. Agric. Food Chem.* **1986**, *34*, 440−444.

Heukeshoven, J.; Dernik, R. Simplified method for silver staining of proteins in polyacrilamide gels and the mechanism of silver staining. *Electrophoresis* **1985**, *6*, 103−112.

Javeri, H.; Wicker, L. Partial purification and characterization of peach pectinesterase. *J. Food Biochem.* **1991**, *15*, 241−252.

Laemmli, U. K. Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Lee, M.; Macmillan, J. D. Mode of action of pectic enzymes. I. Purification and certain properties of tomato pectinesterase. *Biochemistry* **1968**, *7*, 4005−4010.

Lidster, P. D.; Porrit, S. W.; Tung, M. A. Effects of a delay in storage and calcium chloride dip on surface disorder incidence in Van cherry. *J. Am. Soc. Hortic. Sci.* **1979**, *104*, 298−300.

Lin, T.-P.; Liu, Ch.-Ch.; Chen, S.-W.; Wang, W.-Y. Purification and characterization of pectinmethylesterase from *Ficus awkeotsang* Makino Achenes. *Plant Physiol.* **1989**, *91*, 1445−1453.

Manjon, A.; Iborra, J. L.; Romero, C.; Canovas, M. Properties of pectinesterase and endo-D-polygalacturonase coimmobilized in a porous-glass support. *Appl. Biochem. Biotechnol.* **1992**, *37*, 19−31.

MAPA *Norma de Calidad para Cerezas*; Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación: Madrid, Spain, 1987.

Markovič, O.; Jörnvall, H. Pectinesterase. The primary structure of the tomato enzyme. *Eur. J. Biochem.* **1986**, *158*, 455−462.

Markovič, O.; Heinrichová, K.; Lenkey, B. Pectolytic enzymes from banana. *Collect. Czech. Chem. Commun.* **1975**, *40*, 769−774.

McFeeters, R. F.; Armstrong, S. A. Measurement of pectin methylation in plant cell walls. *Anal. Biochem.* **1984**, *139*, 212−217.

Moustacas, A.; Nari, J.; Borel, M.; Noat, G.; Ricard, J. Pectin methylesterase, metal ions in plant cell-wall extension. The role of metal ions in plant cell-wall extension. *Biochem. J.* **1991**, *279*, 351−354.

Nari, J.; Noat, G.; Ricard, J. Pectin methylesterase, metal ions and plant cell-wall extension. Hydrolysis of pectin by plant cell-wall pectin methylesterase. *Biochem. J.* **1991**, *279*, 343−350.

Pitkaenen, K.; Heikinheimo, R.; Pakkanen, R. Purification and characterization of *Erwinia chrysanthemi* B374 pectin methylesterase produced by *Bacillus subtilis*. *Enzyme Microb. Technol.* **1992**, *14*, 832−836.

Pressey, R.; Woods, F. M. Purification and properties of two pectinesterases from tomatoes. *Phytochemistry* **1992**, *31*, 1139−1142.

Randhawa, J. S.; Dhillon, B. S.; Bal, J. S.; Bhullar, J. S. Studies on the pectin methylesterase activity during cold storage of pathernakh pear. *J. Food Sci. Technol.* **1987**, *24*, 71−73.

Rexová-Benková, L.; Markovič, O. Pectic enzymes. *Adv. Carbohydr. Chem. Biochem.* **1976**, *33*, 323−385.

Rhodes, M. J. C. The maturation and ripening of fruit. In *Senescence in Plants*; Thimann, K. V., Ed.; CRC Press: London, UK, 1980.

Ricard, J.; Noat, G. Electrostatic effects on the dynamic of enzyme reactions at the surface of plant cells. 1. A theory of the ionic control of a complex multi-enzyme system. *Eur. J. Biochem.* **1986**, *155*, 183−190.

Sedmak, J. J.; Gossberg, S. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G-250. *Anal. Biochem.* **1977**, *79*, 544−552.

Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Wei, Ch.; Marshall, M. R. Purification and properties of pectinesterases of marsh white grapefruit pulp. *J. Agric. Food Chem.* **1991a**, *39*, 1080−1085.

Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Wei, Ch.; Marshall, M. R. Stability of pectinesterases of marsh white grapefruit pulp. *J. Agric. Food Chem.* **1991b**, *39*, 1075−1079.

Taillan, E.; Ambid, C.; Pech, J. C.; Raynal, J. Demethylation of pectic substances: relationship to methylesterase activity during brine storage of cherries. *J. Food Sci.* **1992**, *57*, 682−685.

van Buren, J. P. Pectic substances of sweet cherries and their alteration during SO_2 brining. *J. Food Sci.* **1967**, *32*, 435−436.

Versteeg, C.; Rambouts, F. M.; Spaansen, C. H.; Pilnik, W. Thermostability and orange juice cloud destabilizing properties of multiple pectinesterase from orange. *J. Food Sci.* **1980**, *45*, 969−972.

Wegrzyn, T. F.; McRae, E. A. Pectinesterase, polygalacturonase, and β-galactosidases during softening of ethylene-treated kiwi fruit. *HortScience* **1992**, *27*, 900−902.

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