Capillary Electrophoresis Analysis of Orange Juice Pectinesterases

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Pectinesterase (PE) was extracted from orange juice and pulp with 1 M NaCl, desalted, and separated using capillary electrophoresis (CE) gel procedures (CE-SDS-CGE) and isoelectric focusing (CE-IEF). PE resolved as a single peak using noncoated fused silica columns with CE-SDS-CGE. CE-IEF separation of PE required acryloylaminoethoxyethanol-coated columns, which had limited stability. Thermal stability of PE extracts before and after heating at 75 °C for 30 min and at 95 °C for 5 min established heat labile and heat stabile fractions with identical PE migration times by CE-SDS-CGE or CE-IEF. Peak magnitude decreased to a constant value as heating time increased at 75 °C. Regression analysis of CE-SDS-CGE peak migration times of molecular weight (MW) standards estimated both heat labile and heat stable PE at MW ≈36 900. Traditional SDS-PAGE gel separation of MW standards and active PE isolated by IEF allowed estimation of MW ≈36 000. CE-SDS-CGE allowed presumptive, but not quantitative, detection of active PE in fresh juice.

Keywords: *Pectinesterase; citrus; orange juice; capillary electrophoresis*

INTRODUCTION

Thermal treatment of commercial citrus juices to inactivate 99% of pectinesterase (PE) requires temperatures ≥90 °C for 1 min and results in some flavor changes (*1*). If juice is maintained fresh and not heat-stabilized, PE activity results in the perceived defect of cloud/serum separation. This defect may also occur during storage if the enzyme is only partially inactivated. Because of this problem and the economic importance of citrus juices, the thermal and chemical properties of citrus PE have been studied extensively since early literature reports concerning its activity (*2*), extraction, and characterization (*3*). Thermal inactivation studies of citrus PE also led researchers to recognize the existence of thermally labile (TLPE) and thermally stable (TSPE) forms of this enzyme.

Characterization and purification of citrus PE from fruit tissue has involved salt extraction, activity measurements, gel filtration, molecular weight (MW) estimation, gel electrophoresis, and isoelectric focusing (IEF). Studies of limes and oranges suggested two major PE forms, which were similar in MW (*4*). In lemons, two purified forms were also estimated to have similar MW (35 and 33 kDa) and isoelectric points of >11 and 9, respectively (*5*). Gel filtration purification of PE from navel orange revealed a high MW (54 kDa) TSPE and two low MW (36 kDa) TLPE forms (*6*). These multiple PE forms were further characterized by their high isoelectric points and were separated by pH gradient electrophoresis, determining that TSPE accounted for ∼6−10% and the TLPE ∼90% of cloud destabilizing activity in juice (*7*). Two forms of PE were also isolated from grapefruit, with TSPE more stable to lower pH than TLPE (*8*). A preparative IEF procedure indicated two fractions with PE activity could be extracted from grapefruit pulp, with favored extraction of TSPE at pH 3.0 (*9*).

The existence of TLPE and TSPE in citrus is generally not argued; however, the number of forms and their molecular weights have not been resolved. Authors have reported two to six forms of PE from various citrus fruits and tissues. Use of anion-exchange, heparin, and gel filtration chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis isolated six forms, including a TSPE of MW 37.5 kDa, and estimated other PE forms to have MW from 34 to 47 kDa (*10*). MW estimation by SDS-PAGE of a TSPE from orange juice reported a value of 42.7 kDa (*11*). Juice cloud loss occurred after addition of four PE forms, which included one TSPE, isolated from orange peel (*12*).

The present study shows that capillary electrophoresis (CE), in conjunction with thermal treatments, may be used to monitor active forms of PE in orange juice and tissues. We also report that CE-IEF and CE-SDS−capillary gel electrophoresis (CGE) may be used to estimate the molecular weights of TLPE and TSPE.

MATERIALS AND METHODS

PE Extraction from Finisher Pulp and Juice. PE was extracted from finisher pulp (150 g) recovered from fresh Valencia orange juice by adding 450 g of 1 M NaCl plus 0.1 M Tris, pH 8.0, with HCl. This mixture was homogenized by blending, stirred for 1 h, and centrifuged at 10 000*g* for 20 min. Fermentation was prevented by the addition of 0.02% $NaHSO_3$. The supernatant was filtered through Miracloth (Calbiochem, La Jolla, CA), ammonium sulfate was added to 75% saturation, and the mixture was stirred overnight. A pellet recovered after centrifugation at 10 000*g* was solubilized in 50 mL of 10 mM Na_2PO_4, pH 7.0, buffer and then centrifuged at 10 000*g* for 10 min. The supernatant was membrane-dialyzed (SpectraPor No. 3, Fisher Scientific, Atlanta, GA) twice for 8 h each against 2 L of 10 mM Na_2PO_4, pH 7.0, buffer. Recovery of dialysate was ∼50 mL, which was centrifuged at

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10 000*g* for 10 min. These procedures were performed at 4 °C to preserve enzyme activity. The final supernatant protein concentration was 1.4 mg/mL (Coomassie protein assay, Pierce, Rockford, IL). This procedure was representative of the PE extractions performed for the various procedures of this study. For CE-SDS-CGE analysis, the PE extract could be directly desalted by passing the sample through a column (PD-10, Amersham Pharmacia Biotech, Piscataway, NJ).

PE extraction of single-strength juice for CE-SDS-PAGE or CE-IEF required centrifuging 100−150 mL of juice (10 000*g*, 20 min) to obtain a pellet (8−10 g) containing enough active enzyme for analytical procedures. The CE procedures required less sample than the above preparative method, so the pellet was treated as follows: extraction (room temperature, 1 h) with 25 mL of 1 M NaCl adjusted to pH 8.0 with Tris base (*9*), stirring for 1 h, centrifuging at 10 000*g* for 20 min, desalting with 2.5 mL of supernatant with a PD-10 column, rinsing, recovering 3.5 mL of solution, centrifuging at 10 000*g* for 5 min, and then filtering with 0.45 μm filters, prior to CE analysis. Thermal treatments of these extracts were performed before desalting.

Thermal Inactivation and Activity Measurement. Samples (25 mL) of the PE extract dialysates were maintained as controls, and 25 mL was heated between 2 and 45 min at 75 °C in a water bath. A thermocouple determined when samples reached set-point temperatures for timing. Heating time depended on the experimental situation. Total PE inactivation was achieved by heating at 95 °C for 5 min. After ice bath cooling, samples and controls were centrifuged at 22 000*g* and 4 °C to remove any denatured protein before assay for PE activity.

PE activity was determined by titration as follows: Samples for assay (10 μL) were added to 5 mL of 0.2% citrus pectin (Sigma P-9135) in 0.075 M NaCl adjusted to pH 7.7 by the addition of 0.1 M and then with 0.004 M NaOH. When the pH decreased to 7.5, 0.004 M NaOH was added dropwise to maintain the pH at 7.5 for ~4.0 min. The actual time was recorded and the milliliters of 0.004 M NaOH measured. The PE activity (units) was calculated variously as microequivalents per minute per gram of pulp (from the dialysate/pulp ratio), microequivalents per minute per milliliter of sample, or specific activity as microequivalents per minute per milligram of protein, depending on the sample measured.

CE-IEF. Desalted PE dialysates in 10 mM Na_2PO_4, pH 7.0, were prepared for CE-IEF as follows: For 2 mL samples, 110 μL of Pharmalyte pH 3−10 plus 16 μL of TEMED and 400 μL of 1% methylcellulose (η = 1500 cp at 2%) (Sigma M-0387) were added to 1.50 mL of PE dialysate. The mixture was vortexed well, and then 1 mL was pipetted into a microvial (15 × 45 mm, Fisher Scientific), placed into the CE instrument (P/ACE 2100 with System Gold Software, Beckman Instruments, Fullerton, CA). The column (50 μm × 27 cm) was coated with acryloylaminoethoxyethanol (AAEE) (Bio-Rad, Hercules, CA). The detector monitored peaks at 280 nm. The column was prerinsed for 5 min with the sample and then focused for 10 min at +15 kV, 25 °C, with 20 mM H_3PO_4 as anolyte and 20 mM NaOH as catholyte. The focused zones were mobilized chemically by exchanging the catholyte with 20 mM NaOH plus 80 mM NaCl and applying 15 kV.

CE-SDS-CGE. Desalted PE dialysates were adjusted to a concentration of 1% SDS and 5% 2-mercaptoethanol, by directly adding these reagents, followed by boiling (10 min) and filtering through 0.45 μm filters. Sample vials were placed in the instrument and pressure-injected for 45 s. Separation conditions were as follows: column was 75 μm × 27 cm noncoated fused silica, run buffer was from the CE/SDS Protein Kit (Bio-Rad), 25 °C, −10 kV (polarity reversed), detection at 214 nm. Approximate CE run times were 15 min, with the PE peak retention near 10 min. For MW estimation, a MW standard mixture containing 100 ppm of lysozyme (14.4 kDa) chymotrypsinogen A (25 kDa), carbonic anhydrase (30 kDa), ovalbumin (45.5 kDa), bovine serum albumin (66.2 kDa), and phosphorylase B (97.4 kDa) (Promega Corp., Madison, WI) was co-injected with the PE extract. Linear regression analysis of the MW versus retention time was used to obtain a best estimate of the MW of the PE peak.

IEF. PE was extracted from juice finisher pulp as described above, assayed for activity and protein content. One sample was heated at 75 °C for 30 min to inactivate the TLPE, and the other was used as a nonheated control. IEF of heated and nonheated extracts was performed with a Rotofor cell (Bio-Rad) using Pharmalyte ampholytes (pH 8−10.5) using the procedure in the Rotofor manual. Focusing took ~4 h. Fractions were collected, and the pH of each of 20 fractions was measured. Fractions were dialyzed twice with SpectraPor No. 3 membranes for 8 h each against 2 L of 10 mM Na_2PO_4, pH 7.0, buffer. After dialysis, the PE activity of fractions 5−20 was determined. Active fractions (9−20) were analyzed by CE-SDS-CGE and traditional SDS-PAGE.

IEF of control and heated samples was performed to obtain combined active fractions, which could be concentrated and used in a preparative gel procedure allowing recovery of a band (MW ~36000 kDa). Combined fractions (20 mL) of an IEF run of heated extracts were concentrated by ultrafiltration (PM 10 membrane, model 8050 Amicon stirred cell unit, Fisher Scientific) to 2.5 mL. This sample was analyzed by preparative SDS-PAGE and stained with zinc, and the 36 000 MW band was cut out of the gel and destained. The staining/destaining procedure was as follows: After electrophoresis, gels were washed with water and stained by gentle shaking for 5 min in 0.3 M $ZnCl_2$. Stain development was stopped by placing gels in water and the band cut out and stored in a freezer. The stained bands were destained three times in 0.25 M EDTA−0.25 M TRIS, pH 9.0, with gentle agitation. The protein band was collected from the destained gels by electroelution (Bio-Rad 422 Elector-eluter) following the procedure in the manual. The samples (~1 mL) were dialyzed (SpectraPor No. 3 membranes) three times against 2 L of water and stored in a freezer for CE-SDS-CGE or SDS-PAGE analysis. This procedure was repeated twice to obtain enough protein (~50 mg/run) for subsequent analyses.

SDS-PAGE. SDS-PAGE analysis of control and heated sample fractions from IEF was performed and compared with the MW standards. SDS-PAGE gel concentration (12%) was prepared and samples analyzed as described in the instruction manual for the Mini-Protean II Dual Slab Cell (Bio-Rad). Band detection was by silver stain (0.2% $AgNO_3$).

RESULTS AND DISCUSSION

Assays of orange juice for PE activity have traditionally involved titration procedures, similar to the above method. Generally, these methods are useful, simple, and quantitative when applied to the raw materials. PE studies for biochemical isolation, purification, and kinetic purposes require more complex protein analytical procedures, particularly gel electrophoresis. Capillary electrophoresis may be used to perform presumptive tests of PE activity in juice and tissue samples, as well as to obtain rapid high-resolution separations of the TLPE and TSPE forms present in extracts of the enzyme. The following discussion considers applications of CE for analytical study of citrus PE extracts by comparison with traditional protein separations.

PE Extract Preparation. Concentrated enzyme samples required for biochemical PE analysis necessitate performing extractions from tissue or juice. The pulp fraction separated by finishing from juice (*1*) represents the edible tissue, which contains PE associated with cloud destabilization. Although PE is also present in the peel, it is very unlikely that enzyme from this fruit component will contribute significantly to activity in the juice fraction. Release of bound PE enzymes from insoluble tissue is accomplished by salt extractions. Extractions should be performed at pH 7−8 for proper study of both heat-labile and heat-stable PE

Table 1. PE Activity of Valencia Orange Pulp Extracts Heated at 75 °C for Various Times

min at 75 °C	activity (μequiv/min/mL)[a]
0	181 ± 13[b]
2	9.0
5	8.5
10	8.6
20	7.2
30	9.0
45	8.6

[a] For calculation of specific activity, protein concentration at 0 min = 1.06 mg/mL, for 2−45 min = 0.75 mg/mL. [b] Values represent triplicate studies, duplicate analyses, for 2−45 min pooled SD = 0.6.

Table 2. PE Activity and pH of IEF Fractions from Typical Unheated Controls and Extracts Heated at 75 °C for 30 min

	activity (μequiv/min/mL)[b]		
IEF fraction[a]	control	heated	pH
5	0	0	8.1
6	0	0	8.3
7	0	0	8.4
8	0	0	8.6
9	0	0	8.8
10	0	0	8.9
11	24	0	9.1
12	46	0	9.2
13	74	1	9.3
14	185	3	9.5
15	296	4	9.7
16	217	4	9.9
17	216	3	10.3
18	40	2	11.3
19	0	0	12.0
20	0	0	12.5

[a] Ampholyte was pH 8−10.5. [b] For calculation of specific activity, average protein concentration of combined fractions was 1.3 mg/mL for control and 0.8 mg/mL for heated samples for two studies.

forms, because highest activity occurs in extracts at alkaline pH conditions (*9*). The salt must be removed once the enzyme is solubilized. For traditional gel electrophoresis or IEF, replications and sample procedures may need 25−50 mL of final salt-free dialysate of 1−2 mg of protein/mL. Preparation of this amount of PE dialysate and electrophoretic analysis may take 2 days, including study of any necessary variables, such as thermal or kinetic treatments. By contrast, PE sample preparation for CE analysis can be simplified to salt extraction, centrifugation, and column desalting, with actual CE analysis time of <20 min/sample.

IEF and SDS-PAGE. To study TLPE and TSPE, dialysates were heated at 75 °C for 30 min to inactivate the TLPE, allowing remaining TSPE activity. For these studies, PE activity was measured by the standard quantitative titration method to verify active enzyme during IEF and electrophoresis procedures. An unheated control sample maintained maximum activity contributed by both enzyme forms in the extracts. Verification of residual TSPE activity in samples heated at 75 °C is presented in Table 1. To ensure that a sample contained TSPE, heating for 30−45 min verified that TSPE activity remained, after inactivation of the TLPE. Typical IEF of unheated control and PE heated at 75 °C for 30 min allowed activity assays and pH measurement of IEF fractions (Table 2). These results indicate that activity is mostly confined to IEF fractions between

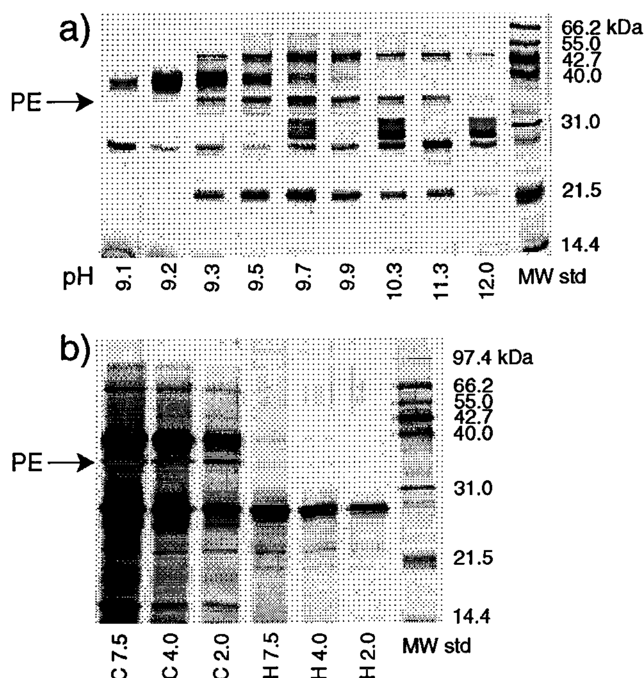

Figure 1. (a) SDS-PAGE 12% gel of individual IEF pH fractions (3.8 μL loading) from extracts heated at 75 °C for 30 min, compared with MW standards. (b) Combined IEF fractions (7.5, 4, and 2 μL loadings) from control (C7.5, C4.0, and C2.0) and 95 °C heated (H7.5, H4.0, and H2.0) extracts.

pH 9 and 10.5 in both control and heated PE extracts and that active enzyme remains after heating at 75 °C.

The IEF study provided several results presented in Figure 1. Selected pH fractions (9−19) of both control and heated samples were combined, and the ampholyte was removed by desalting (PD-10 columns). These samples were run with MW standards as traditional SDS-PAGE for MW determination. Some IEF fractions from the 75 °C heated samples were analyzed by SDS-PAGE, which verified the 36 kDa band remained (Figure 1a). Heating at 95 °C resulted in loss of the 36 kDa band (Figure 1b). Combined fractions 13−18 (20 mL) of two IEF runs of heated extracts were concentrated by ultrafiltration (PM 10 membrane) to 2.5 mL. This concentrated sample was analyzed by preparative SDS-PAGE and stained with zinc, and the 23 and 36 kDa bands were cut out of the gel and destained. Because the 23 kDa band was present after heating at 75 °C, it was speculated that it might be a PE form; however, it did not have PE activity and was of no further interest. The purified 36 kDa band was used to determine the retention time of the 36 kDa peak during CE-SDS-CGE analysis and verification by SDS-PAGE (Figure 2). The 36 kDa band represented the TSPE present after the sample extracts had been heated to 75 °C for 30 min.

CE-IEF. For CE-IEF, the column was rinsed for 5−7 min with the desalted sample or dialysate, focused for 10 min, and then chemically mobilized for detection at 280 nm. A typical electropherogram from nonheated extracts (Figure 3) had a basic PI region to the left and acidic PI to the right, and the peak (labeled PE) was identified as a single peak from traditional IEF fractions with activity. Although resolution of the peaks by CE-IEF was very good, the AAEE columns available have limited stability for PE applications, lasting only for 200−300 injections. For other applications, these col-

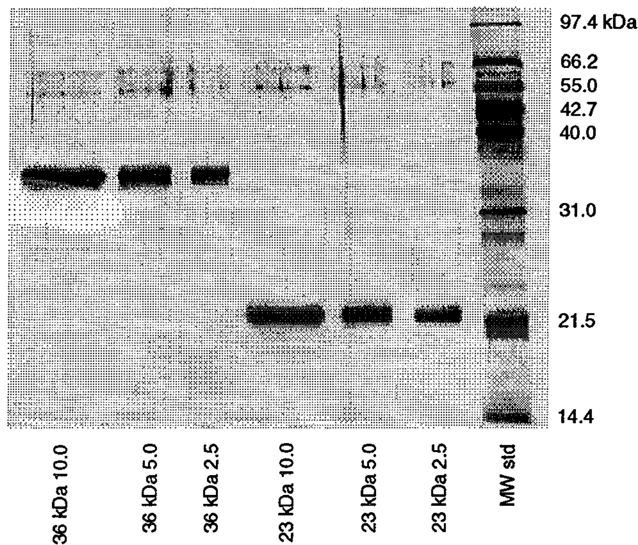

Figure 2. Traditional SDS-PAGE 12% gel (silver stain) of 36 and 23 kDa bands from zinc stained/destained preparative gels of 75 °C heated extracts. Loadings were at 10, 5, and 2.5 μL against midrange MW standards.

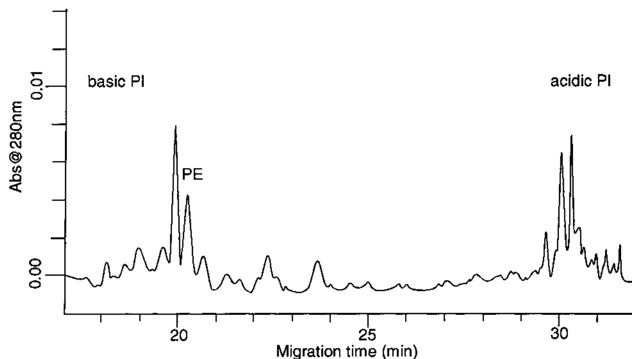

Figure 3. CE-IEF electropherogram indicating the PE peak, basic and acidic PI regions from a desalted nonheated extract. Conditions: ampholyte pH 3–10, 7 min rinse, +15 kV, 25 °C, focus 10 min with 20 mM H_3PO_4 (anolyte), 20 mM NaOH (catholyte), mobilize by replacing catholyte with 20 mM NaOH + 80 mM NaCl; column Bio-Rad AAEE, 50 μm × 27 cm, detection at 280 nm.

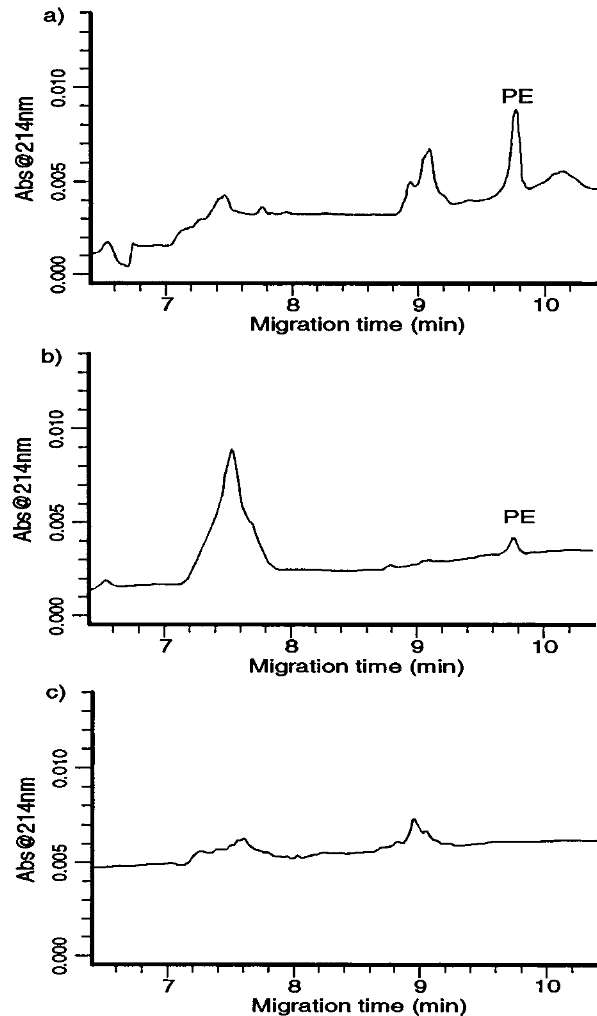

Figure 4. CE-SDS-CGE electropherograms indicating the PE peak from the following extracts: (a) nonheated, (b) heated at 75 °C for 30 min, and (c) heated at 95 °C for 5 min. Conditions: Bio-Rad CE/SDS run buffer, 25 °C, −10 kV (reverse polarity), 45 s pressure injection, 75 μm × 27 cm noncoated fused silica column.

umns have been reported to have good stability (*13*). A less costly column (linear polyacrylamide) gave similar resolution, but was less stable, lasting for only 100 injections in our studies. The pH extremes of the PE separation at the voltage used resulted in the gradual loss of the coating from the column, the primary reason for instability.

CE-SDS-CGE. A challenge to separating PE by CE involves binding of the enzyme to the fused silica columns. Initial trials to separate native active PE on standard fused silica columns were not successful, as the enzyme bound to the column and failed to migrate. Attempts to use columns with hydrophobic or hydrophilic coatings did not give the resolution needed to identify PE without SDS. Eventually, coating chemistry changed, causing instability and loss of the coating, increasing binding of the enzyme to the column. A satisfactory procedure was developed for CE-SDS-CGE, because the detergent essentially renders the molecules to have equal anionic charge, allowing separation according to MW. The gel coating can be replenished each injection with the run buffer from the protein kit.

Peak Identification. Results of CE-SDS-CGE analyses of control, 75 °C heated, and 95 °C heated extracts indicated that only one peak could be identified, which represented PE (Figure 4). This peak was related to PE by both the thermal treatments and by injection of the recovered PE from the preparative separation of the 36 kDa band. Peak height was the estimator of active enzyme in the extracts. The peak height was a maximum in nonheated extracts of pulp and raw juice (Figure 4a), decreasing significantly in extracts heated at 75 °C (Figure 4b) and essentially disappearing in extracts heated at 95 °C (Figure 4c). Similar results were obtained when raw juice was heated, prior to PE extraction. Quantitative measurement of PE activity was not performed by CE procedures.

Molecular Weight. CE procedures allow precise measurement of peak migration times, which was found to be advantageous for MW estimation by comparison with standard MW mixtures. Results of CE-SDS-CGE analysis of recovered PE mixed with MW standards and compared with the pure PE peak are presented in Figure 5. By linear regression analysis, the MW of the peak representing PE was estimated as 36.9 kDa for both TLPE and TSPE. This is in agreement with one

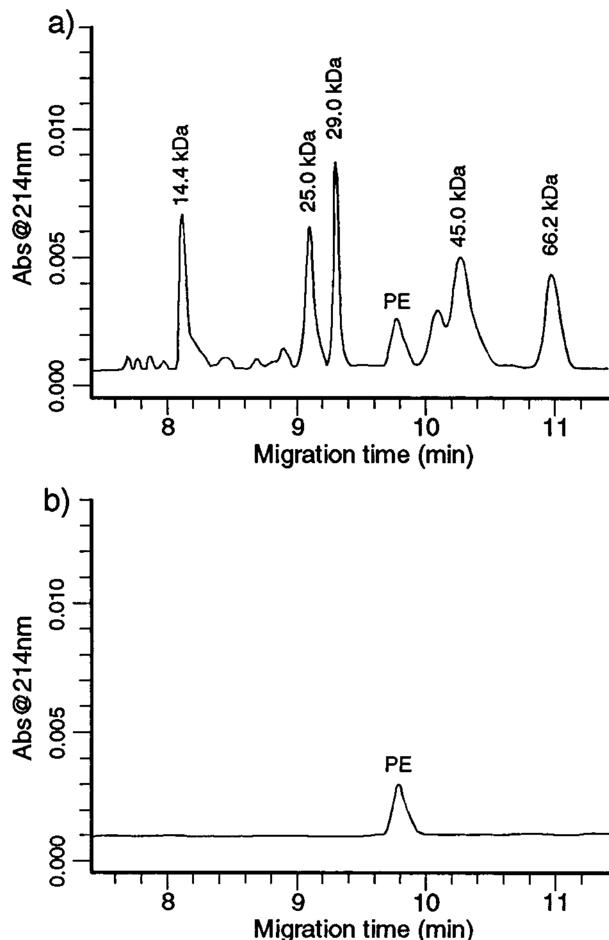

Figure 5. (a) CE-SDS-CGE electropherogram indicating (a) the ~36 kDa PE peak from SDS-PAGE zinc stain samples (Figure 2) and midrange MW standards and (b) CE-SDS-CGE of 36 kDa PE collected from SDS-PAGE. Conditions: Bio-Rad CE/SDS run buffer from the protein kit, 25 °C, −10 kV (reverse polarity), 45 s pressure injection, 75 μm × 27 cm noncoated fused silica column.

study (*5*) but differs with other reports suggesting different molecular weights for forms of the enzyme (*6, 10, 11*).

Conclusions. Use of SDS-PAGE capillary gel electrophoresis simplifies and allows rapid separation of PE from pulp and juice, once the enzyme is extracted and the SDS derivative prepared. Compared with traditional gel electrophoresis, use of this CE technique offers the advantage of excellent separations achieved in <30 min with sample volumes of 2 mL. Although only one sample may be run at a time, instruments containing autoinjectors allow many samples to be analyzed. The data generated are also saved in spreadsheet and graphic formats, allowing statistical evaluation. The estimation that both TLPE and TSPE have the same MW (36.9 kDa) is based on the resolution properties of the CE-SDS-CGE technique and the heating studies, which indicated only one peak having PE activity was affected by the thermal treatments. The presence of this peak in juice extracts is presumptive evidence of active enzyme. However, traditional titrimetric PE assays are quantitative and more time-efficient than CE analysis for detecting active PE in juice.

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