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Effect of Intrinsic and Extrinsic Factors on the Interaction of Plant Pectin Methylesterase and Its Proteinaceous Inhibitor from Kiwi Fruit

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A proteinaceous pectin methylesterase inhibitor (PMEI) was isolated from kiwi fruit (*Actinidia chinensis* cv. Hayward) and purified by affinity chromatography on a cyanogen bromide (CNBr) Sepharose 4B−orange PME column. The optimal pH of banana PME activity was 7.0, whereas that for carrot and strawberry PME activity was 9.0. The optimal pH for the binding between kiwi fruit PMEI and these PMEs was 7.0. The kiwi fruit PMEI has a different affinity for PME depending on the plant source. The inhibition kinetics of kiwi fruit PMEI to banana and strawberry PME followed a noncompetitive type, whereas that to carrot PME followed a competitive type. The kiwi fruit PMEI was mixed with banana, carrot, and strawberry PME to obtain PMEI−PME complexes, which were then subjected to thermal (40−80 °C, atmospheric pressure) or high-pressure (10 °C, 100−600 MPa) treatment. Experimental data showed that the PMEI−PME complexes were easily dissociated by both thermal and high-pressure treatments.

KEYWORDS: Kiwi fruit; *Actinidia chinensis*; pectin methylesterase inhibitor; PMEI; PMEI−PME complex; high pressure; thermal processing

INTRODUCTION

Plant pectin methylesterase (PME) has been described as a cell-wall-bound enzyme capable of catalytically causing beneficial as well as detrimental effects on fruit- and vegetable-based food products. In the beverage industry, this enzyme is known to be responsible for the cloud loss (phase separation) of some fruit and vegetable juices during processing and storage. So far, conventional thermal processing and, to a minor extent, high-pressure processing are the most useful approaches applied to control PME activity (*1*). Apart from these, the discovery of a powerful proteinaceous inhibitor of pectin methylesterase (PMEI) in ripe kiwi fruit (*Actinidia chinensis*) by Balestrieri and co-workers (*2*) created new opportunities to control endogenous PME activity.

According to these authors, the kiwi fruit PMEI, which effectively inhibits PME in the pH range of 3.5−7.5, is specific for PME as it is active against several plant PMEs (PMEs from orange, tomato, apple, banana, and potato), but not against polygalacturonase or amylase. Several authors have attempted to gain insight into the biochemical characterization of this inhibitor. Balestrieri and co-workers (*2*) reported that kiwi fruit PMEI possesses an isoelectric point (p*I*) of 3.5, attributed to the amino acid composition consisting of a very high content of acidic residues. Camardella and co-workers (*3*) pointed out that kiwi fruit PMEI comprises 152 amino acid residues accounting for a molar mass of 16.277 kDa, and a far-UV circular dichroism analysis (CD) spectrum indicated a predominant α-helix conformation in its secondary structure. Camardella and co-workers (*3*) also stated that in kiwi fruit PMEI protein microheterogeneities were detected at five positions of the amino acid sequence (Ala/Ser56, Tyr/Phe78, Ser/Asn117, Asn/Asp123, and Val/Ile142), indicating the presence of several isoforms of the PMEI protein in the fruit. There were also reports about a similarity between kiwi fruit PMEI and plant invertase inhibitors (*4*−*6*). In particular, the four Cys residues, which in PMEI are involved in the disulfide bridges, are conserved. The comparison of the sequence of these inhibitors confirms the existence of a novel class of proteins with moderate but significant sequence conservation, comprising plant proteins acting as inhibitors of sugar metabolism enzymes (*3*).

Another proteinaceous PMEI, which is from jelly fig (*Ficus awkeotsang* cv. Makino), was discovered and purified by Jiang and co-workers (*7*, *8*). As reported, jelly fig PMEI is composed of 57% basic amino acids (including lysine, arginine, and histidine) and possesses a molar mass between 3.5−4.5 kDa.

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However, the p*I* of this PMEI has not been reported yet. Similarly to kiwi fruit PMEI, jelly fig PMEI was described to be active against several plant PMEs (PMEs from tomato, apple, asparagus, guava, pea pod, papaya, orange, and pineapple). The inhibitor showed an extreme thermostability up to 100 °C at neutral pH (*7*).

McMillan and Pérombelon (*9*) discovered a nonproteinaceous PMEI in potato tuber (*Solanum tuberosum* subsp. *tuberosum* cv. Katahdin), which is a heavy side-branched uronic acid chain with a molar mass of ∼200 kDa (detected by gel filtration). This PMEI is extremely thermostable and retained full activity even after incubation at 120 °C for 10 min. It is active against PME from potato as well as different plant species with an uncompetitive mode of action, but it is not active against PME from bacteria and fungi. The inhibition is independent of pH and temperature. The authors also stated that its inhibitory activity was lost after a treatment with two pectin-degrading enzymes, polygalacturonase (PG) and pectin lyase (PnL).

Given the potential application to use PMEI in controlling the PME activity that causes cloud loss in fruit and vegetable juices, studies on the interaction of PME with PMEI and temperature−pressure-processing stability of PMEI are of major importance. In this study, a proteinaceous PMEI was isolated and purified from kiwi fruits. PME crude extracts from a number of plant sources were obtained for a screening experiment in terms of PME−PMEI interaction. Among those PME sources, banana, carrot, and strawberry PME were further purified and used for the rest of the experiments. Banana, carrot, and strawberry PME were selected because they are interesting PME sources; strawberry PME is an extremely pressure stable enzyme, whereas carrot PME is a pressure labile enzyme, and banana is a pressure−temperature stable enzyme.

MATERIALS AND METHODS

Materials. Cucumber (*Cucumis sativa* cv. Pepino Almeria), banana (*Musa acuminata* cv. Cavendish and Chiquita), orange (*Citrus sinensis* cv. Valencia), potato (*Solanum tuberosum* cv. Nicola), carrot (*Daucus carota*), strawberry (*Fragaria ananassa* cv. Elsanta), tomato (*Lycopersicon esculentum* cv. Flandria Prince), and kiwi fruit (*Actinidia chinensis* cv. Hayward) were purchased from local supermarkets. Apple pectin (DE 75%) was from Fluka Chemical Co. (Buchs, Switzerland), CNBr Sepharose 4B from Sigma (St. Louis, MO), and alcohol oxidase (EC 1.1.3.13) from Sigma (Bornem, Belgium). Tris(hydroxymethyl)-aminomethane (referred to as Tris) was from Merck KGaA (Darmstadt, Germany). Other chemicals were of analytical grade.

Kiwi Fruit PMEI Extraction and Purification by Affinity Chromatography. PMEI was extracted from kiwi fruits according to the method of Giovane and co-workers (*10*, *11*) and Ly Nguyen and co-workers (*12*, *13*). About 0.75 kg of ripe kiwi fruits was peeled and homogenized in water (1:1 w/v) at 4 °C. The supernatant containing PMEI activity was separated from the pellet by centrifugation at 20000*g*, 4 °C, for 20 min, adjusted to pH 6.0 using 1 M NaOH, and then filtered using a G3 glass filter to eliminate seeds and other small particles. Kiwi fruit PMEI in the crude extract (the supernatant) was further purified using a single-step affinity chromatography on a CNBr Sepharose 4B−orange PME column prepared following a procedure described by Ly Nguyen and co-workers (*12*, *13*). First, kiwi fruit juice containing PMEI activity was mixed with CNBr Sepharose 4B−orange PME gel for 2 h. Afterward, the matrix was washed with distilled water and packed onto a column (XK16, Amersham Biosciences, Uppsala, Sweden). The column was washed with 75 mL of 2 mM KH_2PO_4 buffer (pH 6.0) containing 0.5 M NaCl. The PMEI was recovered by eluting with 20 mM Na_2CO_3 buffer (pH 9.5) containing 1 M NaCl at a flow rate of 0.1 mL/min. After the elution, fractions containing PMEI activity were pooled and desalted using Centricon Plus-20 (PL-10, Millipore). The purified kiwi fruit PMEI concentrate achieved was dissolved in 20 mM Tris-HCl buffer (pH 7.0), quickly frozen in liquid nitrogen, and stored at −80 °C for all experiments.

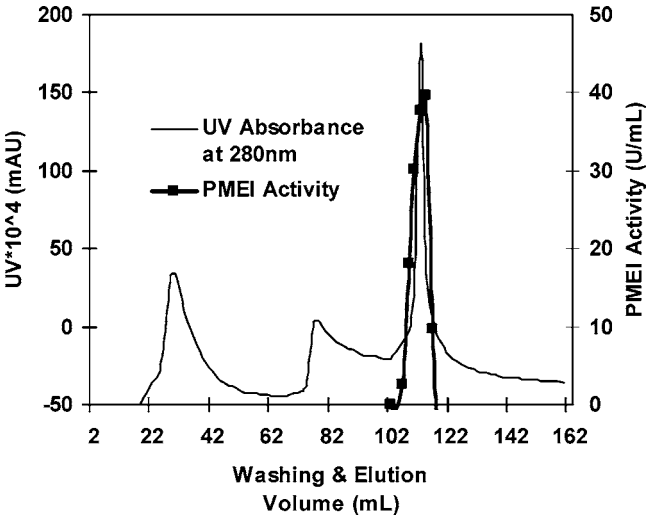

Figure 1. Washing and elution profiles of kiwi fruit PMEI on CNBr Sepharose 4B–orange PME. Washing buffer was 2 mM KH_2PO_4 (pH 6.0) containing 0.5 M NaCl. Elution buffer was 20 mM Na_2CO_3 (pH 9.5) containing 1 M NaCl. UV absorbance was measured at 280 nm.

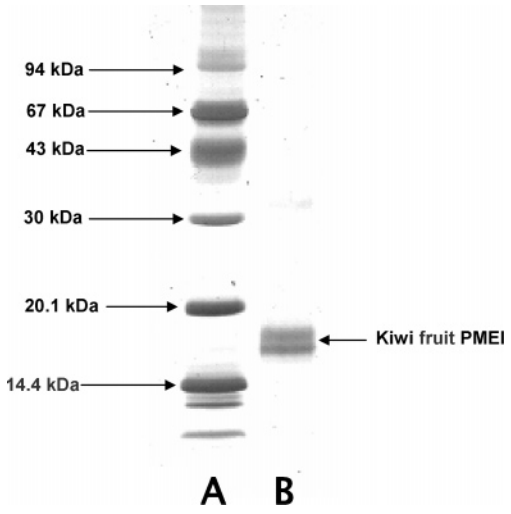

Figure 2. SDS-PAGE (20% homogeneous) of purified kiwi fruit PMEI with silver-stained proteins: (lane A) standard protein markers; (lane B) PMEI.

PME Extraction. The PME extraction procedure described by Ly Nguyen and co-workers (*12*) was followed for carrot and potato, whereas the one by Ly Nguyen and co-workers (*13*) was applied for the rest of the materials. PME crude extracts were dissolved in and dialyzed against 20 mM Tris-HCl buffer (pH 7.0). The dialyzed crude extracts were freeze-dried and stored at −80 °C for further experiments.

PME Purification by Affinity Chromatography. Banana (cv. Cavendish), carrot, and strawberry PME crude extracts were further purified using the same purification technique as applied for kiwi fruit PMEI, that is, single-step affinity chromatography on a CNBr Sepharose 4B−kiwi fruit PMEI column (*12*, *13*). After the elution, fractions containing PME activity were pooled and desalted using Centricon Plus-20 (PL-10, Millipore). The purified banana, carrot, and strawberry PME concentrates achieved were dissolved in 20 mM Tris-HCl buffer (pH 7.0), quickly frozen in liquid nitrogen, and stored at −80 °C for all experiments.

PME Assay. PME activity was measured by continuous recording of the titration of carboxyl groups released from a pectin solution with 0.01 N NaOH using an automatic pH-stat (Metrohm, Herisau, Switzerland). Routine assays were performed with a 3.5 mg mL^{-1} apple pectin solution (DE 75%, 30 mL) containing 0.117 M NaCl at pH 7.0 and 22.5 °C. The activity unit (U) of PME was defined as the amount

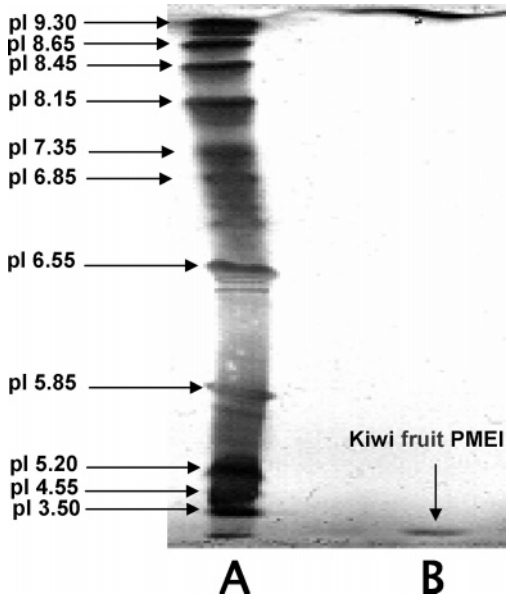

Figure 3. IEF of purified kiwi fruit PMEI: (lane A) standard protein markers; (lane B) PMEI.

of enzyme required to release 1 μmol of carboxyl group per minute, under the aforementioned assay conditions.

PMEI Assay. A sample of an excess amount of PME solution was mixed with a certain amount of PMEI solution and then incubated at 25 °C for 15 min. Afterward, the sample was titrimetrically determined for its residual PME activity (as described under PME Assay). PMEI activity was calculated as the difference between the PME activity of a blank sample (without PMEI) and residual PME activity of the mixture.

Methanol Determination. Methanol content was determined spectrophotometrically according to a method of Klavons and Bennett (*14*). First, methanol was oxidized to formaldehyde with alcohol oxidase, followed by condensation with 2,4-pentanedione to obtain 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (*15*). The colored product was determined spectrophotometrically at 412 nm.

Inhibitory Capacity of Purified Kiwi Fruit PMEI to PME of Different Sources. PME crude extracts (of cucumber, banana, orange, strawberry, tomato, potato, and carrot) (in 20 mM Tris-HCl buffer, pH 7.0, 12 units/mL) were mixed (1:1 v/v) with purified kiwi fruit PMEI (in 20 mM Tris-HCl buffer, pH 7.0, 0.09 mg/mL) and incubated at 25 °C for 15 min. Samples of 250 μL of PME−PMEI mixtures and blanks (PME−Tris-HCl buffer) were evaluated for residual PME activity using the routine PME assay. Data were reported on the basis of the mean of two measurements.

Effect of pH on Inhibitory Capacity of Purified Kiwi Fruit PMEI to Purified Banana, Carrot, and Strawberry PME. PME activity with or without the addition of PMEI as a function of pH was studied. Samples of 250 μL of purified banana, carrot, and strawberry PME (20, 18, and 9 units/mL, respectively) were mixed with 250 μL of purified kiwi fruit PMEI (0.0244 mg/mL) and 20 mL of distilled water in the reaction vessel of an automatic pH-stat equipment for 15 min (22.5 °C) for preincubation at different pH values ranging from 4 to 10. Measurement of residual PME activity of samples thereafter was performed at pH values of preincubation at 22.5 °C. The PME activity of blank samples (mixtures without PMEI) was also determined using the same experimental conditions. Data reported were based on the mean of two measurements.

PME Inhibition Kinetics of Purified Kiwi Fruit PMEI to Purified Banana, Carrot, and Strawberry PME. PME activity with or without the presence of PMEI as functions of substrate concentration (0.20−3.33 mg/mL pectin with the presence of 0.117 M NaCl) was investigated. Samples of 250 μL of purified banana, carrot, and strawberry PME (in 20 mM Tris-HCl buffer, pH 7.0; 20, 18, and 9 units/mL, respectively) were mixed with 250 μL of purified kiwi fruit PMEI (in 20 mM Tris-HCl buffer, pH 7.0) of different protein

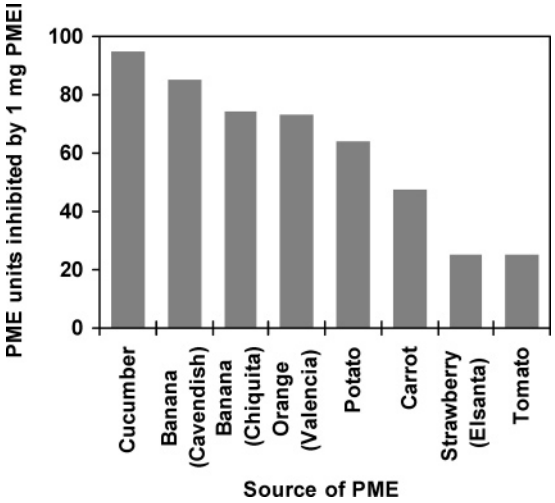

Figure 4. PME inhibitory capacity of purified kiwi fruit PMEI to different sources of PME.

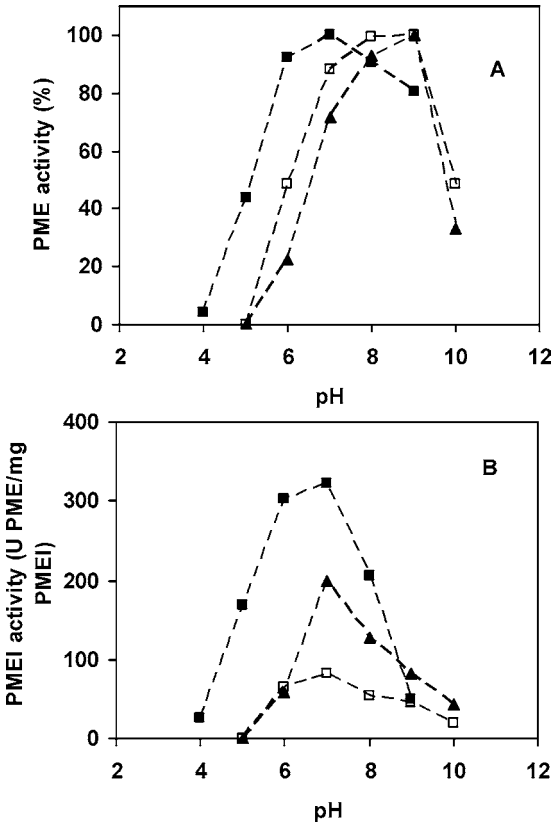

Figure 5. Effect of pH on (**A**) purified banana (■), carrot (▲), and strawberry (□) PME activities and (**B**) inhibitory capacity of purified kiwi fruit PMEI to purified banana (■), carrot (▲), and strawberry (□) PMEs. Symbols represent data points; dashed lines represent linear interpolation.

concentrations for 2 h prior to measurement of residual PME activity. Data reported were based on the mean of two measurements.

Effect of Temperature and Pressure on the Stability of the PME−PMEI Complex. Purified banana, carrot, and strawberry PMEs were mixed with an excess amount of purified kiwi fruit PMEI at 25 °C (pH 7.0) for at least 2 h for complete inhibition of PME activity. The complex was then frozen in liquid nitrogen and stored at −80 °C for experiments. The effect of temperature (40−80 °C) and pressure (100−600 MPa) on the stability of the PME−PMEI complexes was investigated using the automatic pH-stat and high-pressure equipment, respectively. For thermal experiments, pectin samples (30 mL of 3.5 g/L containing 0.117 M NaCl) were first placed in the reaction vessel and controlled for its preset temperature prior to being mixed with 0.3

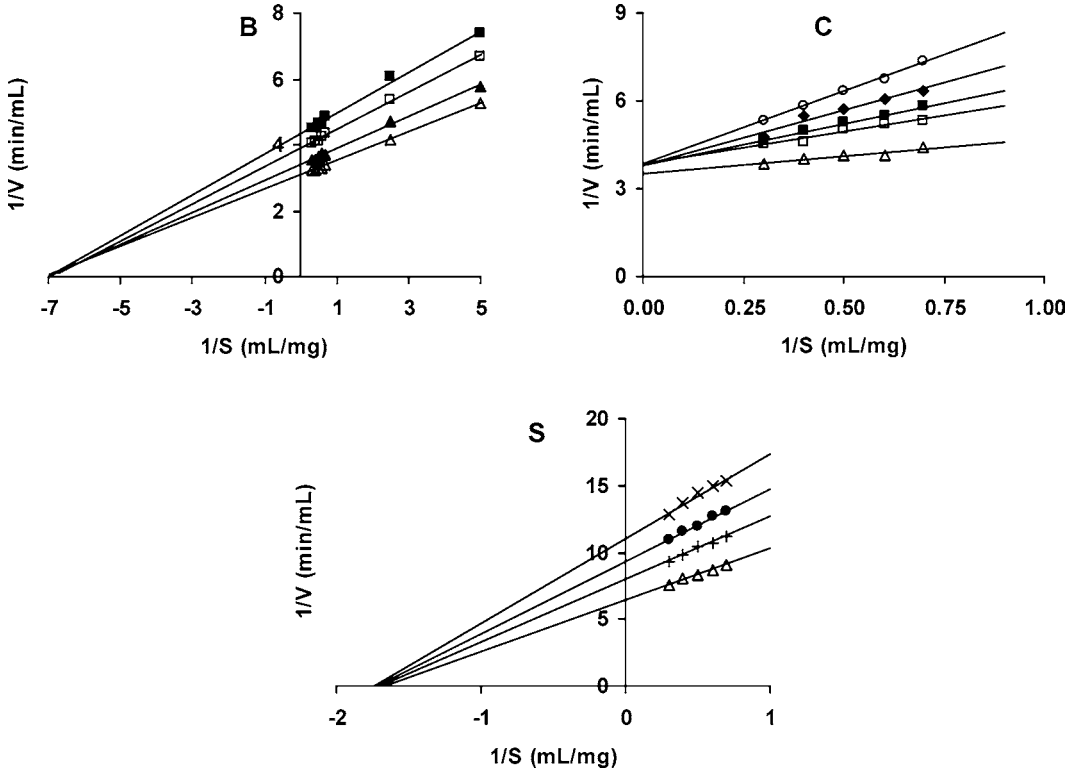

Figure 6. Lineweaver–Burk plots of the inhibition of purified banana PME (**B**), carrot PME (**C**), and strawberry PME (**S**) by purified kiwi fruit PMEI [0.00 (△), 0.86 (▲), 1.72 (□), 2.58 (■), 3.31 (+), 3.45 (◆), 4.31 (○), 6.63 (●), and 9.94 nM (×)] at different substrate concentrations. Symbols represent experimental data points; solid lines represent the best fit based on linear regression.

Table 1. Type of Inhibition of Banana, Carrot, and Strawberry PMEs by Kiwi Fruit PMEI

purified PME	type of inhibition	K_i[a] ± SD	K_m[b] ± SD
banana	noncompetitive	6.32 ± 0.19	0.1458 ± 0.0047
carrot	competitive	0.91 ± 0.17	0.2365 ± 0.0478
strawberry	noncompetitive	14.37 ± 0.21	0.6010 ± 0.0267

[a] K_i (nM) is the dissociation constant. [b] K_m (mg/mL) is the Michaelis–Menten constant.

mL of PME−PMEI complex for the PME assay. The effect of heat on the dissociation of the PME−PMEI complex was quantified by the amount of 0.01 N NaOH consumed to neutralize the −COOH formed. The assay was performed at pH 7.0.

High-pressure experiments were conducted in a multivessel, high-pressure apparatus (six vessels of 40 mL) (Resato, Roden, The Netherlands), which allows pressurization up to 750 MPa in combination with temperatures ranging from −20 to 100 °C. The pressure medium was a glycol−oil mixture (TR 15, Resato). First, 30 mL of pectin solution (4 g/L, containing 6.85 g/L NaCl and 0.1 M Tris, pH 7.0) was mixed with 0.3 mL of PME−PMEI complex and placed into 0.3-mL flexible microtubes (Elkay, Leuven, Belgium), which were then enclosed in the pressure vessels, already equilibrated at the preset temperatures. The time to prepare the samples and to enclose them in the pressure vessels was standardized to 15 min. Pressure was built up slowly using a standard pressurization rate of ∼100 MPa/min to minimize the temperature rise due to adiabatic heating (*16*, *17*). After pressure buildup, an equilibration period of 3 min to allow the temperature of the pressure medium to evolve to its preset value (input value) was taken into account (*18*). After 3 min of equilibration, pressure vessels were decompressed in time and the corresponding samples were immediately immersed in a water bath at 85 °C for 2 min to stop residual PME activity prior to placing them in ice−water. The methanol content formed of the samples was determined according to the method of Klavons and Bennett (*14*).

RESULTS AND DISCUSSION

Kiwi Fruit PMEI Purification and Characterization. Kiwi fruit PMEI was eluted with a high ionic strength and high-pH buffer [20 mM Na_2CO_3 buffer (pH 9.5) containing 1 M NaCl]. A single, sharp peak of proteins and PMEI activity was obtained (**Figure 1**). The maximum binding capacity of the CNBr Sepharose 4B−orange PME column was ∼1150 PMEI units (based on the use of crude orange PME for the routine PMEI assay).

Analysis of the purified kiwi fruit PMEI by SDS-PAGE showed a double band of ∼16.9 kDa (**Figure 2**). The molar mass observation obtained in this study is in good agreement with the finding of Giovane and co-workers (*10*). The existence of the double band in SDS-PAGE was attributed to protein microheterogeneities of the PMEI amino acid sequence as observed by Camardella and co-workers (*3*). On isoelectric focusing (IEF), one protein band of pI < 3.5 was observed (**Figure 3**), which is similar to the finding of Balestrieri and co-workers (*2*).

Inhibitory Capacity of Kiwi Fruit PMEI to PME of Different Plant Sources. Kiwi fruit PMEI has a different affinity for PME depending on the plant source. The inhibitor showed the strongest affinity to cucumber PME as 1 mg of PMEI could inhibit ∼95 units of cucumber PME. The affinity was lower to banana PME (84.98 U/mg for cv. Cavendish and 74.52 U/mg for cv. Chiquita), orange PME (73.19 U/mg), potato PME (63.96 U/mg), carrot PME (47.37 U/mg), strawberry PME (25.28 U/mg), and tomato PME (25.02 U/mg), as shown in **Figure 4**. Plant PMEs with different pI values were differently charged at the experimental pH (7.0) and, therefore, showed different affinities to kiwi fruit PMEI. In terms of three-dimensional structure, plant PMEs with the active site cleft different in depth would interact with kiwi fruit PMEI at

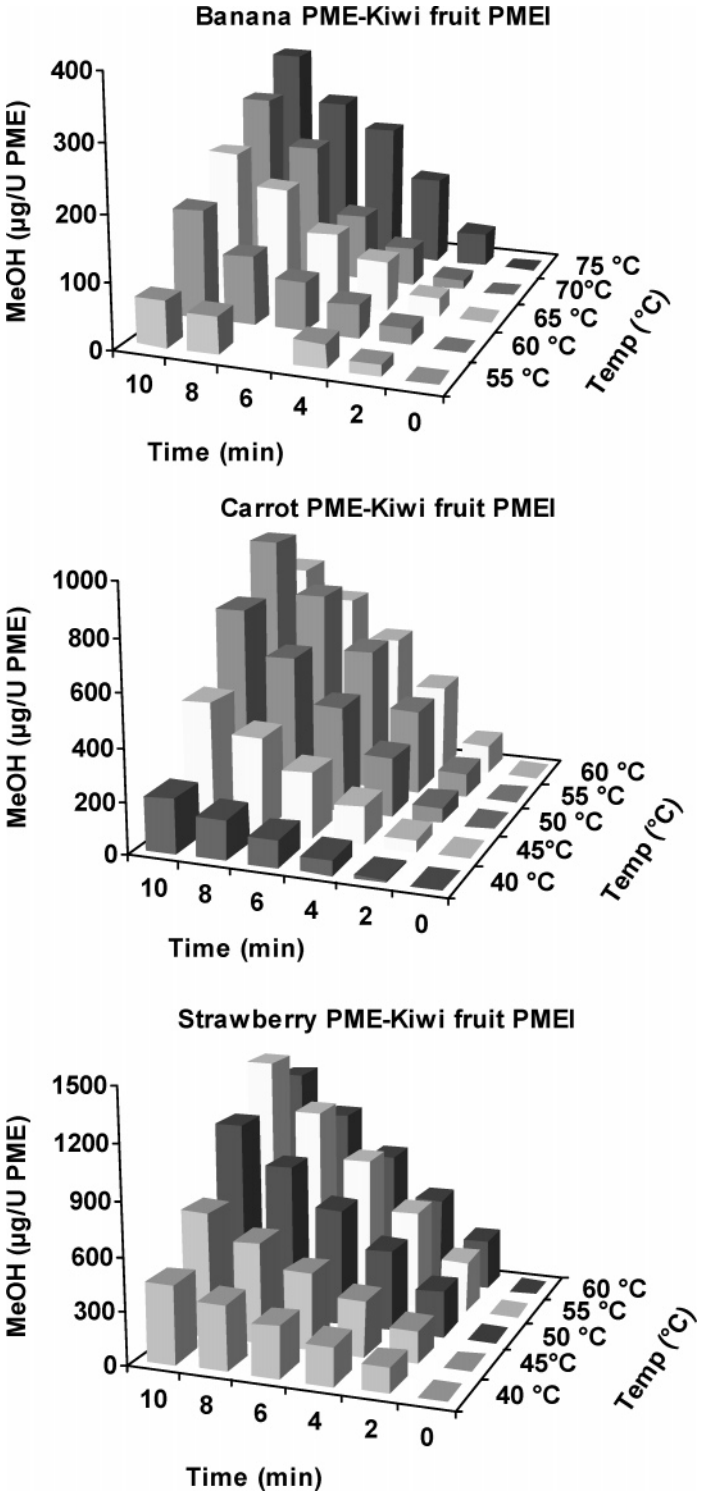

Figure 7. Methanol content formed as a function of temperature and time due to the dissociation of banana, carrot, and strawberry PME–kiwi fruit PMEI complexes. Methanol content formed was calculated for one unit of PME forming the complex.

different levels: the narrower and deeper the active site cleft, the more difficult the PME interacts with the PMEI (*19*).

These findings are in contrast with those observed by Jiang and co-workers (*20*), especially for the affinity between jelly fig PMEI and tomato or orange PMEs. The differences between kiwi fruit and jelly fig PMEIs in terms of molar mass (16 vs 3.5−4.5 kDa) and amino acid composition (amino acid composition of kiwi fruit PMEI consists of a very high content of acidic residues, whereas that of jelly fig PMEI consists of a high content of basic residues) would be an explanation among the others for the differences observed.

Effect of pH on the Interaction between PMEI and PME. The optimal pH for purified banana PME activity was 7.0, wheres that for carrot and strawberry PME activities was 9.0 (**Figure 5A**). Ly Nguyen and co-workers (*12*, *13*, *21*) reported p*I* values ranging from 4.0 to 8.9 for banana PME and a p*I* > 9.0 for carrot and strawberry PMEs. These differences in p*I* values would be a possible explanation, among the others, for the difference of the optimal pH values. The pH value of the environment has also a profound influence on the inhibitory capacity of kiwi fruit PMEI. Purified kiwi fruit PMEI showed the strongest affinity to all three sources of PME investigated

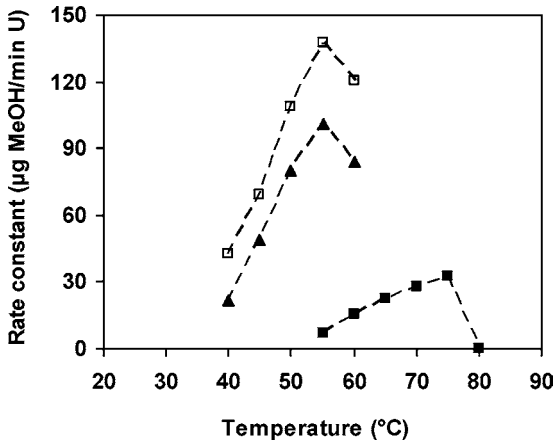

Figure 8. Effect of temperature on the formation rate constant of methanol due to the dissociation of (■) banana, (▲) carrot, and (□) strawberry PME–PMEI complexes. Symbols represent data points; dashed lines represent linear interpolation.

at pH 7.0. However, banana PME was inhibited to a larger extent compared to carrot and strawberry PMEs (**Figure 5B**); this observation is in line with results obtained under Inhibitory Capacity of Kiwi Fruit PMEI to PME of Different Plant Sources.

PME Inhibition Kinetics of Purified Kiwi Fruit PMEI. The inhibition mode of kiwi fruit PMEI on carrot PME was competitive, indicating that the inhibitor binds near or at the PME active sites and that the carrot PME−PMEI complex is unable to bind substrate. The inhibition mode of kiwi fruit PMEI on banana and strawberry PMEs was noncompetitive as shown in **Figure 6** and **Table 1**. Marquis and Bucheli (*22*) also reported that the interaction of tomato PME with kiwi fruit PMEI followed a noncompetitive inhibition type (using apple pectin). Dissociation constants (K_i) for these three cases were calculated as 6.32, 0.91, and 14.37 nM for the inhibition of PMEI on banana, carrot, and strawberry PME, respectively. For competitive inhibition, the K_m is changed by a factor of (1+ [I]/K_i) ([I] is the PMEI concentration) with no change in V_{max}. In the case of noncompetitive inhibition, V_{max} is changed by a factor of 1/(1 + [I]/K_i), whereas there is no change in K_m. The very small K_i (0.91 nM) estimated for the interaction between kiwi fruit PMEI and carrot PME indicated very tight binding between the two proteins, approximately 16- and 7-fold differences in the affinity compared to that between kiwi fruit PMEI and strawberry and banana PMEs. However, the kiwi fruit PMEI bound very tightly to all three aforementioned PMEs in comparison with its competitive inhibition to kiwi fruit PME, where the K_i was 0.22 μM as reported by Balestrieri and co-workers (*2*), who used citrus pectin as substrate. The Michaelis−Menten constants (K_m) estimated for the inhibition kinetics (**Table 1**) are in agreement with those found by Ly Nguyen and co-workers (*12*, *13*, *21*).

Effect of Temperature and Pressure on the Dissociation of PME−PMEI Complex. Temperature and pressure treatments influenced strongly the complexes of banana, carrot, and strawberry PMEs with kiwi fruit PMEI. At atmospheric pressure, there was an increase in product formation as the temperature of thermal treatment was increasing, due to the dissociation of PME−PMEI complexes and the action of PME on the pectin solution (pH 7.0) (**Figure 7**). Reaction rate constants for the

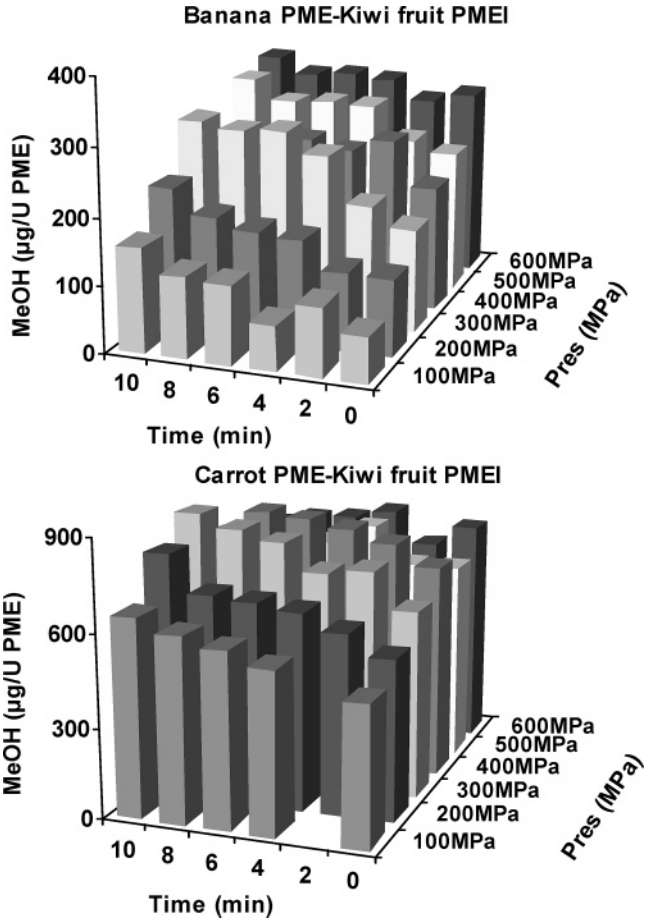

Figure 9. Methanol content formed as a function of pressure and time for the dissociation of banana and carrot PME–kiwi fruit PMEI complexes. Methanol content formed was calculated for one unit of PME to form the complex.

methanol formation were estimated and are plotted in **Figure 8**. For all cases, the rate constants were increasing with temperature increase and decreasing thereafter because of PME heat inactivation (**Figure 8**). The banana PME−kiwi fruit PMEI complex was dissociated only at higher temperatures compared to carrot PME− and strawberry PME−kiwi fruit PMEI complexes. The maximum rate constant for the methanol formation was 32.6 μg/(min·U) compared to 101.1 μg/(min·U) for carrot PME− and 137.8 μg/(min·U) for strawberry PME−kiwi fruit PMEI dissociation.

Similarly, pressure treatment at 10 °C caused the dissociation of banana and carrot PME−PMEI complexes. At 100 MPa, methanol formation was observed, indicating that the PME−PMEI complexes started to dissociate even at mild pressure (**Figure 9**). At time zero, there was already a large amount of methanol formed due to the dynamic pressure−temperature conditions, which were excluded from the time scale. The data obtained showed that the treatment time and the pressure levels favor the methanol formation. For treatments at increasing pressure levels, there was an increase in methanol formation rate constant to a maximum and then a decrease therefrom due to the pressure inactivation of PME. The maximum methanol formation rate constants for both banana and carrot PME−PMEI dissociation were 14.3 and 25 μg/(min·U), respectively, achieved by the treatments at 300 MPa (**Figure 10**). Obviously, it was more difficult to dissociate the banana PME−PMEI complex compared to that of carrot PME.

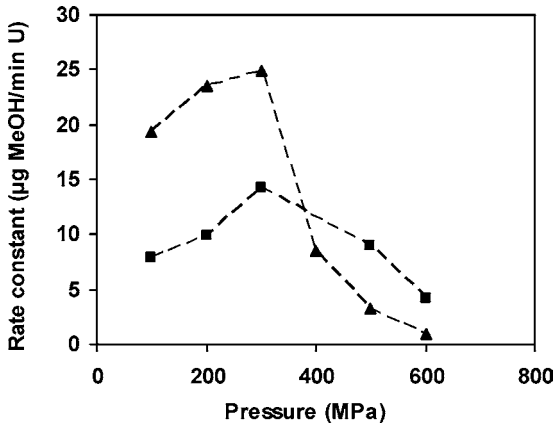

Figure 10. Effect of pressure on the formation rate constant of methanol as consequence of the dissociation of (■) banana and (▲) carrot PME–PMEI complexes. Symbols represent data points; dashed lines represent linear interpolation.

Conclusion. Plant PMEs showed different affinities for kiwi fruit PMEI and ranked from cucumber PME (with the highest affinity for kiwi fruit PMEI among the others) to banana PME, orange PME, potato PME, carrot PME, strawberry PME, and tomato PME. At ambient temperature, banana PME showed the highest activity at pH 7.0, whereas carrot and strawberry PME optimally acted at pH 9.0; all three PMEs showed the highest affinity for kiwi fruit PMEI at pH 7.0. Kiwi fruit PMEI presented a competitive inhibition to carrot PME and a noncompetitive inhibition to banana and strawberry PME. The PME−PMEI complexes formed by kiwi fruit PMEI and banana, carrot, and strawberry PME were very sensitive to the temperature and pressure treatments; they dissociated when treated at 40 °C at atmospheric pressure or at 10 °C at 100 MPa.

LITERATURE CITED

(1) Ludikhuyze, L.; Van Loey, A.; Indrawati; Smout, C.; Hendrickx, M. Effects of combined pressure and temperature on enzymes related to quality of fruits and vegetables: from kinetic information to process engineering aspects. *Crit. Rev. Food Sci. Nutr.* **2003**, *43* (5), 527−586.

(2) Balestrieri, C.; Castaldo, D.; Giovane, A.; Quagliuolo, L.; Servillo, L. A. Glycoprotein inhibitor of pectin methylesterase in kiwi fruit (*Actinidia chinensis*). *Eur. J. Biochem.* **1990**, *193*, 183−187.

(3) Camardella, L.; Carratore, V.; Ciardiello, M. A.; Servillo, L.; Balestrieri, C.; Giovane, A. Kiwi protein inhibitor of pectin methylesterase. Amino acid sequence and structural importance of two disulfide bridges. *Eur. J. Biochem.* **2000**, *267*, 4561−4565.

(4) Greiner, S.; Krausgrill, S.; Rausch, T. Cloning of a tobacco apoplasmic invertase inhibitor. Proof of function of the recombinant protein and expression analysis during plant development. *Plant Physiol.* **1998**, *116*, 733−742.

(5) Fuglevand, G.; Phillips, W.; Mozzanega, P.; Corley, S.; Chengappa, S.; Shields, R. Mapping of tomato genes associated with sugar metabolism. *Tomato Genet. Co−Op Rep.* **1998**, *48*, 22−23.

(6) Scognamiglio, M. A.; Ciardiello, M. A.; Tamburrini, M.; Carratore, V.; Rausch, T. L.; Camardella, L. The plant invertase inhibitor shares structural properties and disulfide bridges arrangement with the pectin methylesterase inhibitor. *J. Protein Chem.* **2003**, *22*, 363−369.

(7) Jiang, C. M.; Lai, Y. J.; Chang, W. H.; Wu, M. C.; Chang, H. M. Pectinesterase inhibitor in jelly fig (*Ficus awkeotsang* Makino) achenes. *J. Food Sci.* **2001**, *66*, 225−228.

(8) Jiang, C. M.; Li, C. P.; Chang, J. C.; Chang, H. M. Characterization of pectinesterase inhibitor in jelly fig (*Ficus awkeotsang* Makino) achenes. *J. Agric. Food Chem.* **2002**, *50*, 4890−4894.

(9) McMillan, G. P.; Pérombelon, M. C. M. Purification and characterization of a high pI pectin methylesterase isozyme and its inhibitor from tubers of *Solanum tuberosum* subsp. *tuberosum* cv. Katahdin. *Physiol. Mol. Plant Pathol.* **1995**, *46*, 413−427.

(10) Giovane, A.; Balestrieri, C.; Quagliuolo, L.; Castaldo, D.; Servillo, L. A glycoprotein inhibitor of pectin methylesterase in kiwi fruit. Purification by affinity chromatography and evidence of a ripening-related precursor. *Eur. J. Biochem.* **1995**, *233*, 926−929.

(11) Giovane, A.; Laratta, B.; Loiudice, R.; Quagliuolo, L.; Castaldo, D.; Servillo, L. Determination of residual pectin methylesterase activity in food products. *Biotechnol. Appl. Biochem.* **1996**, *23*, 181−184.

(12) Ly Nguyen, B.; Van Loey, A. M.; Fachin, D.; Verlent, I.; Indrawati; Hendrickx, M. E. Partial purification, characterization, thermal and high-pressure inactivation of pectin methylesterase from carrots (*Daucus carrota L.*). *J. Agric. Food Chem.* **2002**, *50*, 5437−5444.

(13) Ly Nguyen, B.; Van Loey, A.; Fachin, D.; Verlent, I.; Indrawati; Hendrickx, M. Purification, characterization, thermal, and high-pressure inactivation of pectin methylesterase from bananas (cv. Cavendish). *Biotechnol. Bioeng.* **2002**, *78*, 683−691.

(14) Klavons, J. A.; Bennett, R. D. Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. *J. Agric. Food Chem.* **1986**, *34*, 597−599.

(15) Wood, P. J.; Siddiqui, I. R. Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. *Anal. Biochem.* **1971**, *39*, 418−428.

(16) Weemaes, C. A.; De Cordt, S. V.; Ludikhuyze, L. R.; Van den Broeck, I.; Hendrickx, M. E.; Tobback, P. P. Influence of pH, benzoic acid, EDTA, and glutathione on the pressure and/or temperature inactivation kinetics of mushroom polyphenoloxidase. *Biotechnol. Prog.* **1997**, *13*, 25−32.

(17) Ludikhuyze, L.; Indrawati; Van den Broeck, I.; Weemaes, C.; Hendrickx, M. Effect of combined pressure and temperature on soybean lipoxygenase. 1. Influence of extrinsic and intrinsic factors on isobaric−isothermal inactivation kinetics. *J. Agric. Food Chem.* **1998**, *46*, 4074−4080.

(18) Van den Broeck, I.; Ludikhuyze, L. R.; Van Loey, A. M.; Hendrickx, M. E. Inactivation of orange pectinesterase by combined high-pressure and temperature treatments: a kinetic study. *J. Agric. Food Chem.* **2000**, *48*, 1960−1970.

(19) D'Avino, R.; Camardella, L.; Christensen, T. M. I. E.; Giovane, A.; Servillo, L. Tomato pectin methylesterase: Modeling, fluorescence, and inhibitor interaction studies-Comparison with the bacterial (*Erwinia chrysanthemi*) enzyme. *Proteins: Struct., Funct., Genet.* **2003**, *53*, 830−839.

(20) Jiang, C. M.; Li, C. P.; Chang, H. M. Influence of pectinesterase inhibitor from jelly fig (*Ficus awkeotsang* Makino) achenes on pectinesterases and cloud loss of fruit juices. *J. Food Sci.* **2002**, *67*, 3063−3068.

(21) Ly Nguyen, B.; Van Loey, A. M.; Fachin, D.; Verlent, I.; Duvetter, T.; Vu, T. S.; Smout, C.; Hendrickx, M. E. Strawberry pectin methylesterase (PME): Purification, characterization, thermal and high-pressure inactivation. *Biotechnol. Prog.* **2002**, *18*, 1447−1450.

(22) Marquis, H.; Bucheli, P. Inhibition of tomato pectin methylesterase by partially purified kiwi pectin ethylesterase inhibitor protein. *Int. J. Food Sci. Technol.* **1994**, *29*, 121−128.

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