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Characterization of Pectinesterase Inhibitor in Jelly Fig (*Ficus awkeotsang* Makino) Achenes

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Pectinesterase inhibitor (PEI) extract prepared from intact jelly fig (*Ficus awkeotsang* Makino) achenes was separated by membrane (MWCO 3 and 10 kDa) and fractionated by a Sepharose G-50 gel permeation chromatography. Results from Sepharose G-50 gel permeation chromatography and concanavalin A Sepharose chromatography revealed PEI as polypeptides with molecular weights ranging from 3.5 to 4.5 kDa. Incubation of a PE (1 unit/mL)−PEI (2 mg/mL) mixture for 1 min decreased the PE activity by ∼50%. On the basis of the results of Lineweaver−Burk double-reciprocal plots, Michaelis constant (K_m) and V_{max} values for jelly fig achenes PE (pH 6.0, 30 °C) were 0.78 mM $-OCH_3$ and 1.09 μequiv of −COOH/min, respectively. In addition, PEI competitively inhibited both citrus and jelly fig achenes PEs.

KEYWORDS: ***Ficus awkeotsang*** **Makino achenes; pectinesterase; pectinesterase inhibitor; competitive inhibition**

INTRODUCTION

Pectinesterases (pectin pectyl-hydrolase, EC 3.1.1.11) (PEs) catalyze the de-esterification of pectin and convert the protopectin to soluble pectin and pectate. De-esterification of pectin by PEs from higher plants, such as tomato, is blockwise-type, whereas that of pectin by PEs from microorganisms and by alkali is random-type (*1*, *2*).

Jelly fig (*Ficus awkeotsang* Makino) is a native woody vine that grows on hillsides 800−1800 m high in Taiwan. Jelly curd from the water extract of jelly fig achenes in the presence of calcium is locally popular as a summer drink. Lin et al. (*3*) indicated that PE from jelly fig achenes is a monomer of polypeptide with a molecular weight of 38 kDa and an isoelectric point (p*I*) of 3.5. However, Komae et al. (*4*) stated that this enzyme is a polypeptide with a molecular weight of 42 kDa and a p*I* of 4.5. The very low p*I* is supposed to be one of the features of jelly fig achene PE that is different from other PEs from higher plants (*5*, *6*). The PE from jelly fig achenes functions optimally at pH 6.5 in 0.1−0.7% NaCl (*7*).

The PE inhibitors (PEIs) are substances that tend to decrease the PE-catalyzed reaction rate. Hydrolysis products (such as polygalacturonic acid) from pectin by PE and sugars (such as sucrose) exhibited noncompetitive inhibition on the activity of PEs from papaya and banana sources (*5*, *8*). Some polysaccharides (∼200 kDa) consisting of mainly uronic acids in potato displayed uncompetitive inhibition on potato PE, and the inhibition was independent of pH value and temperature (*9*). The presence of chemicals such as potassium iodide and sodium dodecyl sulfate also reduced PE activity remarkably (*10*, *11*). Recently, a glycoprotein with a molecular weight of 28 kDa was purified from kiwi fruit, which exhibited remarkable inhibition on the activity of PEs from tomato, orange, apple, banana, and potato sources (*12*). The inhibition mode of such glycoprotein on kiwi PE was proved to be competitive (*12*), whereas that of glycoprotein on tomato PE was noncompetitive (*13*). In addition, this purified glycoprotein was immobilized to separate and determine the residual pectin methylesterase activity in thermally treated vegetable products (*14*, *15*).

Previously, Jiang et al. (*16*) reported that PE activity in the solution from intact achenes increased gradually and then reduced to about zero during the 90 h extraction of PE. PE activity of jelly fig achenes was almost eliminated when crushed achenes in the homogenized achene solution were used throughout the same PE extraction procedure. Therefore, some substances released from crushed achenes during PE extraction were considered to be responsible for the inhibitory effect of PE activity (*16*). On the basis of the above findings, more basic data regarding the characterization of PEI from jelly fig achenes are needed before it could be considered for possible utilization in the food industry.

Therefore, a project was designed for the isolation, purification, and characterization of PEI from jelly fig achenes. Kinetic studies of PEs from citrus and jelly fig achenes were also

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performed in the presence or absence of PEI to understand the inhibition mode of PEI on PEs.

MATERIALS AND METHODS

Preparation of PE from Jelly Fig Achenes. Five grams of dry and intact achenes was gently stirred in a 150 mL beaker with 75 mL of 4% NaCl at room temperature for 2 h and then allowed to rest at 4 °C for 22 h to obtain the solution after filtration through six layers of cheesecloth. To the collected solution was added ammonium sulfate powder to reach a 100% saturated concentration. After being allowed to rest overnight at 4 °C, the solution was centrifuged (16300*g*, 60 min, 4 °C) to obtain the precipitates, which were then dissolved in 0.15 M NaCl/10 mM Tris-HCl (pH 7.5) and dialyzed (4 °C) for ~24 h against 50 volumes of the same buffer solution to prepare the crude jelly fig PE solution. Subsequently, a CM-Sepharose CL-6B ion-exchange chromatograph, as previously described by Jiang et al. (*11*), was used to obtain the isolated jelly fig achenes PE for kinetic study. The residual achenes were used for PEI preparation.

Purification of PEI from Jelly Fig Achenes. The PE-removed achenes from the above crude PE preparation procedure were previously rinsed twice with 20 volumes (v/w) of distilled water (Milli-Q System, Millipore, Osaka, Japan). Then, the achenes were homogenized (cycle blender, 2 min) and extracted with 15 volumes (v/w) of distilled water for 6 h, followed by centrifugation (20000*g*, 50 min, 4 °C) to obtain the supernatant (crude PEI solution).

Subsequently, membrane (MWCO 3 and 10 kDa) separation was conducted by an Amicon apparatus to obtain three fractions (<3, 3−10, and >10 kDa) from the crude PEI solution. The 3−10 kDa fraction thus obtained was further fractionated by a Sephadex G-50 column (1.6 × 80 cm) chromatography, using distilled water as eluent at a flow rate of 30 mL/min, to pool the fraction inhibiting PE activity. Absorbance at 280 and 490 nm (phenol−sulfuric acid method) was monitored by a spectrophotometer (Helios Alpha, Spectronic Unican, London, U.K.). The PE activity in the presence of PEI was determined with the method described below. The molecular weight of PEI was calculated from a standard curve ($r = 0.99$; $y = -0.00002292x + 0.9263$; $x =$ MW of protein kit; $y = K_{av}$) obtained by plotting K_{av} versus the molecular weight of protein molecular weight markers (chymotrypsinogen A, 25 kDa; ribonuclease A, 13.7 kDa; aprotinin, 6.5 kDa; insulin A, 2.5 kDa; Pharmacia, Uppsala, Sweden).

Amino Acid Analysis of PEI. PEI (0.1 mg) purified by Sephadex G-50 chromatography was mixed with 4 mL (v/w) of 6 N HCl and then incubated at 110 ± 2 °C for 24 h at a reduced pressure (10 mmHg) to prepare the acid hydrolysates. Later, hydrolysates were vacuum-dried with the aid of an aspirator in a rotary evaporator (N-N, Rikakikai Co., Tokyo, Japan) and redissolved in distilled water. The same procedure was repeated until HCl was completely removed. These hydrolysates in 0.1 M citrate−HCl buffer (pH 2.27) were then applied to amino acid analysis (type 6300, high-performance amino acid analyzer, Beckmam, Palo Alto, CA).

Concanavalin A (Con A) Sepharose Chromatography. Swelled Con A gel (Pharmacia) was packed in a column (1.0 × 8.5 cm) and then equilibrated with at least 3 column volumes of eluent A (1 mM $CaCl_2$/1 mM $MnCl_2$/ 0.15 M NaCl/2 mM Tris-HCl buffer, pH 7.5) at a flow rate of 30 mL/h. The eluate was collected (3.0 mL/tube) with a fraction collector (model 203, Gilson, Villiers-leBel, France). Two milliliters (5 mg/mL) of fraction c in the Sephadex G-50 chromatogram was applied to the column that was first eluted with 38 mL of eluent A, followed by the same volume of eluent B (0.5 M glucose containing eluent A, pH 7.5). Absorbance at 280 and 490 nm (phenol−sulfuric acid method) was monitored by a spectrophotometer.

Proteolytic Treatment of PEI. PEI (1 mg/mL) in fraction c of the Sephadex G-50 chromatogram was incubated (enzyme/substrate = 1/10, w/w) with trypsin (1000−2000 units/mg) (T 7409, Sigma Chemical Co., St. Louis, MO) at room temperature (28 ± 2 °C) for 1 h before reaction with citrus PE (1 unit/mL). Addition of 0.1 mL of distilled water to 1.0 mL of PE solution was used as blank.

Kinetic Studies. All kinetic studies were performed under standard enzyme assay conditions. Michaelis constant (K_m, mM $-OCH_3$) and V_{max} (μequiv of −COOH/min) were calculated from Lineweaver−Burk double-reciprocal plots obtained by plotting $1/V$ versus $1/[S]$ (where V is the reaction rate, μequiv of −COOH/min; and [S] is the substrate level, 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5 mM $-OCH_3$). Pectin (degree of esterification = 68%) (Sigma) was used as substrate to react with PEs (1.0 unit/mL) from citrus source (Sigma) and jelly fig achenes at 30 °C, pH 6.0. For the inhibition study, measurements were carried out with and without the mixing (30 °C) of 1 mL of 0.5 or 1.0 mg of PEI/mL with 1 mL of PE for 10 min prior to PE activity assay as described below.

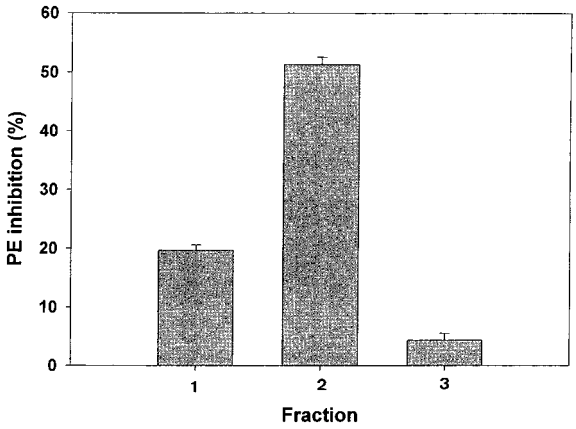

Figure 1. Inhibition (percent) of citrus PE (1 unit/mL) by various PEI (1 mg/mL) membrane (MWCO, 3 and 10 kDa) fractions from jelly fig achenes: 1, 2, and 3, factions <3, 3–10, and >10 kDa, respectively. Each value is the average of three determinations. Bars in the figure indicate standard variation. PE inhibition (%) = 100% − (residual PE activity in reaction mixture/PE activity in the starting sample) × 100.

Protein Assay. Protein concentrations in solutions containing PE and PE inhibitor were assayed according to the Bradford (*17*) method using Bio-Rad protein assay dye reagent. Bovine serum albumin (0.2−1.4 mg/mL) was used to construct the standard curve.

PE Activity Assay. The PE activity was determined according to the method described by Lee and MacMillan (*18*) with minor modifications. To 15 mL of 0.1 M NaCl/0.5% citrus fruit pectin (degree of esterification = 68%) (Sigma) solution (30 °C) with the pH brought to 6.5 immediately before assay was added 0.5 mL (1.0 unit/mL) of enzyme solution. In the determination of the effect of PEI on inhibiting PE activity (residual PE, %), 0.5 mL of a mixture of 1 mL of PEI (2 mg/mL) and 1 mL of PE solution was incubated for 10 min at room temperature before reaction at 30 °C with the substrate solution. The activity of PE was measured by titrating (pH M83 Autocal pH meter, TTT 80 titrator, ABU 80 autoburet; Radiometer, Copenhagen, Denmark) the free protons dissociated from the free carboxyl groups formed by the PE activity. The volumes (milliliters) of 0.01 N NaOH consumed to maintain a pH of 6.0 of the reaction solution at 30 °C (in a water bath) were recorded within a certain reaction time (5 min). An activity unit represents 1 μequiv of the free carboxyl groups produced by the PE hydrolytic activity on the pectin substrate per minute at 30 °C. An enzyme solution previously heated in boiling water for 5 min was used as a blank. PE inhibition (%) = 100% − (residual PE activity in reaction mixture/PE activity in the starting sample) × 100. Each value is the average of three determinations.

RESULTS AND DISCUSSION

Isolation of PE Inhibitor. Pectin and PE were previously removed in NaCl solution from jelly fig achenes, and then the obtained residual achenes were homogenized with distilled water to extract PEI, followed by centrifugation to collect supernatant and membrane (MWCO, 3 and 10 kDa) separation to obtain the PEI-active fraction. As shown in **Figure 1**, the 3−10 kDa fraction (fraction 2) (1.0 mg/mL) exhibited ~52% PE inhibition, higher than that of fraction 1 (20%) and fraction 3 (8%). In a previous paper (*16*), the PE activity in 4% NaCl solution

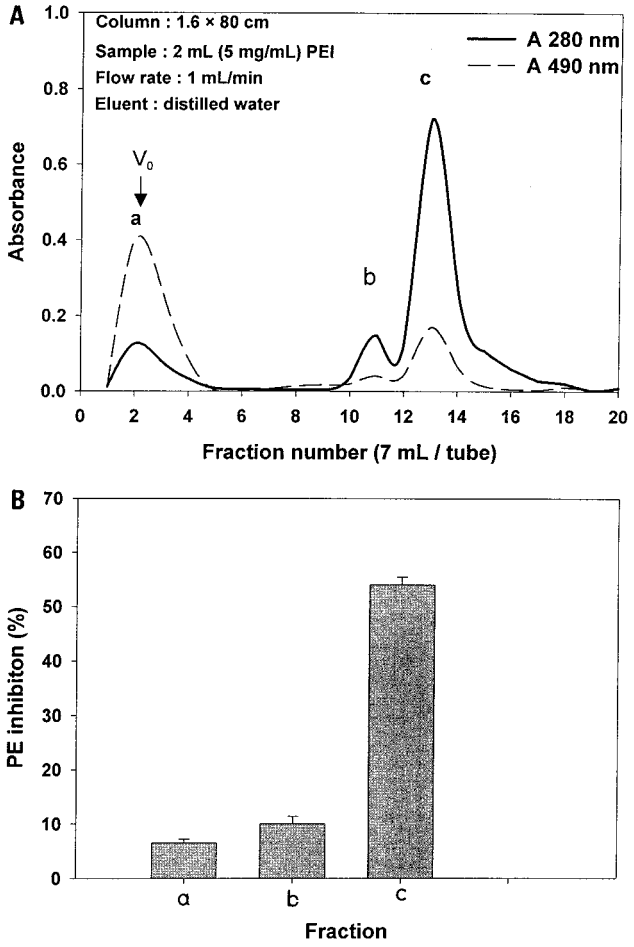

Figure 2. Elution pattern (A) of PEI on Sephadex G-50 chromatography and the PE inhibition (percent) (B) of the pooled fractions (a–c). PEI (3–10 kDa) from fraction 2 in **Figure 1** was applied to the column. V_0 was determined with Blue Dextran 2000. The phenol–sulfuric acid method (A_{490nm}) was used to determine the sugar content in the eluate. Each value is the average of three determinations. Bars in the figure indicate standard variation. PE inhibition (%) = 100% – (residual PE activity in reaction mixture/PE activity in the starting sample) × 100.

dropped remarkably from a maximal value (~12 units/mL) to ~0.2 unit/mL during the 90 h extraction of PE. Apparently, some substances released from achenes inhibited the PE activity (*16*).

The obtained fraction 2 was further fractionated by Sephadex G-50 chromatography (**Figure 2A**), and the PE inhibitions (percent) of the polled fractions (a−c) were compared. It was obvious that the low molecular weight fraction (fraction c) markedly inhibited the citrus PE activity (1.0 unit/mL) by ~55%, followed by fraction b (10%) and fraction a (7%) at the same concentration (1.0 mg/mL) (**Figure 2B**). Therefore, it was apparent that low molecular weight substances from jelly fig achenes were mostly responsible for the reduction of PE activity. Furthermore, the molecular weight of PEI in fraction c in **Figure 2A** was calculated to be between 3.5 and 4.5 kDa from the curve obtained by plotting K_{av} versus the molecular weight of protein markers (data not shown). Apparently, the molecular weight of PEI from jelly fig achenes was much smaller than that of PEI in kiwi (28 kDa) (*12*) and in potato (200 kDa) (*9*).

Biochemical Nature of PEI. Fraction c (10 mg) in the Sephadex G-50 chromatogram of **Figure 2A** was subsequently applied to a Con A Sepharose chromatography (**Figure 3**). Results showed that absorbance at 280 nm of eluate by eluent A was as high as ~0.7, whereas that of the eluate by eluent B was near 0, revealing no apparent specific affinity between Con A in the gel matrix and sample. In addition, no apparent absorbance at 490 nm of the eluate (fraction 8-14) by eluent B was observed. Therefore, the PEI-active components were considered to be free from containing α-D-glucose and/or α-D-mannose (*19*). Furthermore, 0.1 mg of PEI from fraction c in **Figure 2A** was applied to amino acid analysis, and the results showed that PEI was composed of 57% basic amino acids including lysine, arginine, and histidine (**Table 1**).

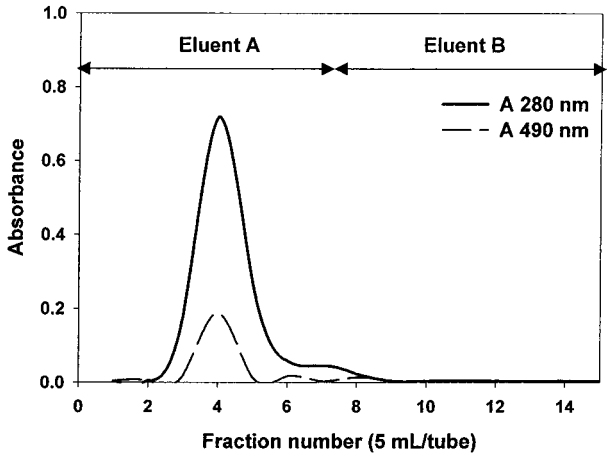

Figure 3. Concanavalin A Sepharose affinity chromatogram of PEI. Pooled fraction C in Sephadex G-50 chromatogram in **Figure 2A** was used as sample.

Table 1. Amino Acid Composition of Jelly Fig Achene PEI (0.1 mg) Purified by Sephadex G-50 Chromatography

type of amino acid	amino acid	concn (nM)	molar percentage[a]
acidic	aspartic acid	0.27	9.9
	glutamic acid	0.34	
basic	lysine	0.13	56.9
	arginine	0.10	
	histidine	3.29	
neutral	serine	0.51	33.2
	threonine	0.15	
	tyrosine	0.02	
	glycine	0.58	
	alanine	0.28	
	isoleucine	0.09	
	phenylalanine	0.06	
	proline	0.08	
	valine	0.11	
	leucine	0.18	
	tryptophan	–[b]	
	cysteine, cystine	–	
	methionine	–	

[a] Concentration (nM) of amino acid/total concentration of compositional amino acids × 100%. [b] Not determined.

The biochemical nature, including amino acid composition and sugar composition, of the glycoprotein inhibitor of pectin methylesterase in kiwi fruit was reported by Balestrieri et al. (*12*). The high content of acidic residues give this glycoprotein a p*I* value of 3.5, whereas the sugar portion is composed of galactose, arabinose, and rhamnose. However, the PEI from potato consists of polysaccharides that are mainly composed of uronic acid (*9*). The high thermal stability of PEI from jelly fig achenes (*16*) was closely related to its biochemical nature of polypeptides as elucidated in the present study (**Figure 2**; **Table 1**).

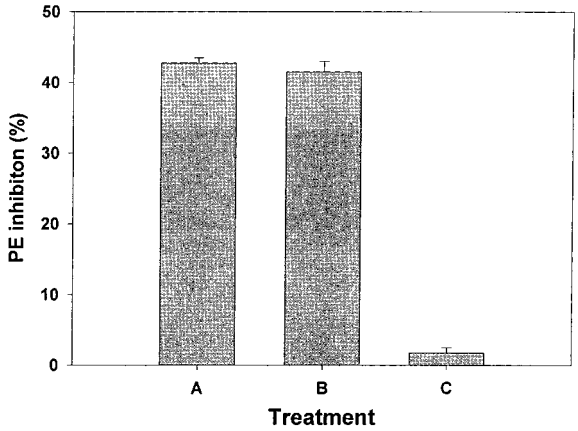

Figure 4. PE inhibition (percent) by PEI (1 mg/mL) reacted with trypsin (10/1, w/w) for 1 h: (A) PEI; (B) PEI + 0.1 mg of distilled water; (C) PEI + 0.1 mg of trypsin. PEI in pooled fraction C in Sephadex G-50 chromatogram was used. Trypsin in PEI solution was thermally treated in a boiling water bath (100 °C) for 10 min prior to PE activity assay. Each value is the average of three determinations. Bars in the figure indicate standard variation. PE inhibition (%) = 100% − (residual PE activity in reaction mixture/PE activity in the starting sample) × 100.

Furthermore, protease was added to pooled fraction c (enzyme/substrate = 1/10, w/w) in the Sephadex G-50 chromatogram in **Figure 2A** to investigate the effect of protein hydrolysis on PEI (**Figure 4**). Apparently, incubation of PEI with trypsin at room temperature for 1 h prior to mixing with PE reduced the PE inhibition to ~3%, revealing that its polypeptide chain contributed to the inhibition of PE activity.

Kinetic Study of PE Inhibitor on PE. The activity of PEs from citrus and jelly fig achene sources as a function of pectin concentration (0.1−2.5 mM −COOH) was investigated. The double-reciprocal plot gave K_m values of 2.22 mM $-OCH_3$ for citrus PE and of 0.78 mM $-OCH_3$ for jelly fig PE (**Figure 5**). All kinetic parameters are the means of three determinations and are reproducible. The K_m value of citrus pE was almost the same as reported. The values for K_m vary widely for plant PEs and vary even within the different varieties of the same fruits and vegetables (*20*). This difference may be related to the presence of PE isozymes and the purity of the PE sample and the substrate used, but it seems that these differences are most probably due to the varietal differences of the fruit samples.

Reaction velocity was determined under standard assay conditions, and a double-reciprocal plot gave V_{max} values of 0.92 μequiv −COOH/min for citrus PE and of 1.09 μequiv −COOH/min for jelly fig PE (**Figure 5**). V_{max} for papaya PE was reported to be 724 μmol/min/mg of PE using pectin with 9.6% methoxyl content as substrate (*8*). Seymour et al. (*21*) indicated that the white grapefruit thermolabile PE exhibited a V_{max} of 724 μmol/min/mg of PE.

V_{max} values for citrus PE and jelly fig achenes PE did not change in the presence or absence of PEI (0.5 and 1 mg/mL). However, K_m increased with the increasing level (0.5 and 1 mg/mL) of PEI in the PE−pectin reaction mixture (**Figure 5**). Lineweaver−Burk plots apparently showed that PEI from jelly fig achenes displayed competitive inhibition on both the PEs from citrus and jelly fig achenes sources, suggesting that PEI and pectin competed for the active sites in PE and the combination of PE and PEI inhibiting the PE activity. Inhibitors are substances that tend to decrease the rate of an enzyme-catalyzed reaction. Lee and MacMillan (*18*) and Lourenco and Catutani (*20*) indicated that polygalacturonic acid competitively inhibited PE activity. Fayyaz et al. (*8*) pointed out that PE from

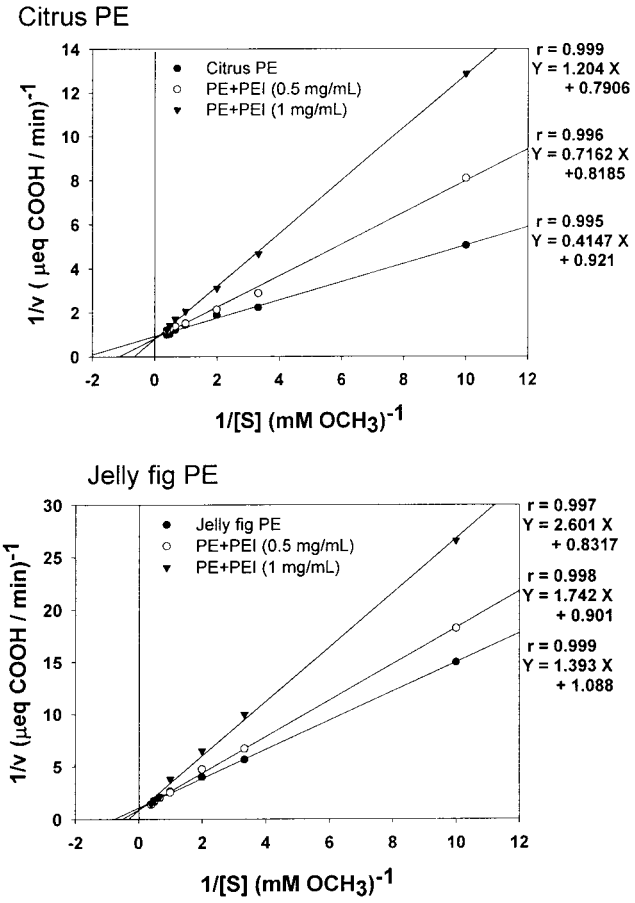

Figure 5. Lineweaver−Burk double-reciprocal plots for PEs (1.0 unit/mL) from citrus and jelly fig sources in the presence or absence of PEI (0.5 mg, 1 mg/mL). A mixture of 1 mL of PE and 1 mL of PEI was incubated at 30 °C for 10 min prior to PE activity assay. Each value is the average of three determinations.

papaya was inhibited by alginic acid through a mixed type of competitive and noncompetitive reaction and by sucrose through an uncompetitive one.

Conclusion. The PEI extract from jelly fig achenes apparently decreased the PE activity. Results from Sephadex G-50 chromatography and Con A Sepharose chromatography revealed that PEI consists of polypeptides in nature with a molecular weight of 3.5−4.5 kDa. The inhibition of this PEI on PEs from citrus and jelly fig sources was determined to be competitive from Lineweaver−Burk double-reciprocal plots obtained by plotting 1/*V* versus 1/[S] in the presence of PEI.

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