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A Novel Method for the Determination of Pectinesterase Inhibitor in Banana

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ABSTRACT

A novel enzyme-linked immunosorbent assay (ELISA) method for the quantification of pectinesterase inhibitor (PEI) in banana was described. The method was based on the specific binding of PE with PEI, rabbit anti-PE polyclonal antibody and goat anti-rabbit IgG-conjugated alkaline phosphatase in series. The ELISA method was found to be more sensitive to quantify PEI in banana, jelly fig (*Ficus awkeotsang* Makino) achene, and kiwi than the conventional pH-stat method. It thus provides a good alternative that saves raw material and efforts in the analysis of PEI in both fresh and processed fruits. The retardation in the ripening of banana pulp in low temperature storage was proven relevant to the maintenance of PEI activity as determined by both methods.

Key words: pectinesterase, inhibitor, pH-stat method, ELISA, postharvest

INTRODUCTION

Pectinesterases (pectin methyl esterases; PEs; E.C. 3.1.1.11) that catalyze the de-esterification and trans-acylation of homogalacturonic acid units of pectins[(1)] are ubiquitous enzymes that can be found in plants, microbial pathogens[(2)] and symbiotic microorganisms during their interactions with plants[(3)]. In plants, PEs play important roles in physiological processes, such as microsporogenesis, pollen growth, seed germination, root development, polarity of leaf growth, stem elongation, fruit ripening and loss of tissue integrity[(4-9)]. The PE is also required for the systemic spread of tobacco mosaic virus through the plants[(10,11)].

PEs can be inhibited by KI and Na-dodecyl sulfate[(12,13)] as well as four kinds of specific inhibitors as follows: 1. some polysaccharides (about 200 kDa) in potato that are consisted of uronic acids mainly[(14)]; 2. a glycoprotein from kiwi fruit that displays remarkable inhibition on PEs from tomato, orange, apple, banana and potato sources[(15)]; 3. a thermal stable PE inhibitor (PEI) from jelly fig achenes that is able to reduce the activities of PEs from citrus, jelly fig achenes, tomato, apple, asparagus and guava[(16)]; 4. an extremely thermostable (80% activity retained after 3 hr in boiling water) PEI found in banana, especially the rubbery fruit[(17)], that acts against banana and pea pod PEs.

Potentially, the most promising application of PEI is to be added to the fresh fruit juice prior to very mild thermal treatment in the production of minimum processed juice at a quality level unachievable by the conventional pasteurization process[(18,19)]. PEI may also be a good additive for winemaking industry to reduce the methanol content in fruit wine[(20)]. Recently, PEI from jelly fig achenes was found to exert a strong inhibition for the growth of human leukemic U937 cells via the following mechanisms: caspase-3 activity upregulating, mitochondria transmembrane potential reduction and apoptosis induction[(21)].

The conventional assay of PE and PEI activities is to employ a pH-stat to monitor the release of protons from carboxylic acid groups, which is resulted from the PE action on pectin molecules. The sensitivity of the pH-stat method is limited by the sluggishness in pH change due to the buffer introduced from sample preparation.

PEI from kiwi fruit (*Actinidia deliciosa*) and PE of tomato (*Lycopersicon esculentum*) were reported to form a complex in a ratio of 1:1[(22-24)]. Therefore, we assume that the ratio of PE and PEI in a complex in other samples is also constant and propose that the change in free PE content as a result of PEI addition could be taken as a measure of PEI content.

Our aim was to establish a novel ELISA method involving the use of anti-PE polyclonal antibody for the assay of PE content and to provide a sensitive and precise alternative for PEI determination. Bananas in postharvest storage were taken as the raw material for experiments.

MATERIALS AND METHODS

I. *Purification of PE from Banana*

PE was purified following the protocols as described

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by Jiang *et al*.[16] and Wu *et al*.[17] with modifications. One kilogram of mature banana fingers (*Musa sapientum L*.; peel color index 3) were peeled, sliced, mixed with 10% NaCl in 1:4 (w/v), and homogenized in a chilled Waring blender for 2 min to obtain homogenate of 2% NaCl. The homogenate was centrifuged at 10,000 ×g for 30 min and the pellet was discarded. The supernatant was fractionated by 30-70% saturation of $(NH_4)_2SO_4$, dialyzed overnight against 0.15 M NaCl/10 mM Tris-HCl (pH 7.5) at 4°C, and concentrated using a Centriprep™ concentrator (MWCO 10 kDa, Amicon). Subsequently, the concentrate was loaded over a CM-Sepharose CL-6B ion-exchange column (2.6 × 40 cm), which was then eluted with 10mM Tris-HCl (pH 7.5) in a NaCl gradient (20 to 800 mM), and a Sephacryl S-200 gel filtration column (1.6 × 95 cm) in 100 mM Tris-HCl (pH 7.5) to collect fractions with PE activity and 280 nm absorbance. The purified banana PE was used as the antigen for anti-PE polyclonal antibody preparation.

II. *Gel Electrophoresis*

The purified PE was also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel following by Coomassie brilliant blue R-250 staining. The low molecular weight protein markers (97, 66, 45, 30, 20.1, and 14.4 kDa) used in the electrophoresis were purchased from Pharmacia.

III. *Preparation of PEI from Banana, Kiwi and Jelly Fig Achenes*

Crude PEIs from kiwi fruit, jelly fig achenes, and banana pulp were prepared according to the methods described by Giovane *et al*.[24], Jiang *et al*.[12], and Wu *et al*.[17], respectively.

IV. *Protein Assay*

Protein concentrations in solutions containing PE or PEI were determined using Bio-Rad protein assay dye reagent (Hercules, CA) with bovine serum albumin as the standard.

V. *Determination of PEI by pH-stat Method*

The activity of PEI was determined according to the method described by Jiang *et al*.[16]. In brief, 0.5 mL of mixture containing crude PEI and citrus PE (1.0 unit/mL; Sigma), which was incubated previously for 10 min at room temperature, was added into 5 mL of 0.1 M NaCl/0.5% citrus fruit pectin (degree of esterification = 68%; Sigma) substrate solution at 30°C (pH 6.5) immediately before assay. The inhibition on the PE-catalyzed formation of free carboxyl groups was evaluated through the titration of the counter ion, H^+, using an autotitrator (pH M83 Autocal pH meter, TTT 80 titrator, ABU 80 autoburette; Radiometer, Copenhagen, Denmark), and taken as a measure of PEI activity. The volume of 0.01 M NaOH needed to maintain the pH of 6.0 of the reaction solution at 30°C for 5 min was recorded. One PEI activity unit represents one μequiv reduction of free carboxyl group production in the pectin substrate per minute.

VI. *Preparation of Anti-PE Polyclonal Antibody*

Anti-PE polyclonal antibody was prepared by immunizing New Zealand white male rabbits intramuscularly with the purified banana PE as described by Tsai and Cousin[25].

VII. *Determination of PEI by ELISA Method*

A sandwich ELISA protocol was applied by referring to Tsai and Cousin[25] and Buchta[26] with modifications. Briefly, 50 μg PEI in 100 μL coating buffer was immobilized overnight on the surface of a 96-well polystyrene microplate at 4°C. Each coated well was washed four times each with 200 μL phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Tween-PBS), blocked with 100 μL of 2% gelatin-PBS at 37°C for 30 min, and rinsed with Tween-PBS. Each rinsed well was added with 100 μL of purified banana PE in coating buffer, incubated at 4°C overnight, and blocked with 100 μL of 0.5% gelatin-PBS at 37°C for 30 min. The washing, blocking and rinsing procedures were repeated once more. Each well was then added with 100 μL of rabbit anti-PE polyclonal antibody in 0.5% gelatin-PBS. The microplate was incubated at 37°C for 90 min. The wells were washed again. Subsequently, 100 μL of goat anti-rabbit IgG: alkaline phosphatase conjugated in 1:2000 dilution was added and allowed to react at 37°C for 90 min. After another washing, 100 μL of Blue-Phos Phosphatase Microwell substrate solution (KPL) was added and incubated at 37°C for 30 min. Finally, 100 μL of 2.5% EDTA was added to terminate the reaction. The absorbance at 650 nm was measured with a microplate reader. The average of six determinations was recorded.

VIII. *Statistical Analysis*

Statistical analysis was performed using SAS Statistical Software, Version 6.11 (SAS Institute). The difference between treatment means was analyzed using General Linear Model Procedure and Duncan's multiple range test. A P value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

I. *Purification of PE from Banana*

The activity of PE in banana pulp was found to be at maximum on the 16th day in a preliminary room temperature storage test (data not shown). Hence, the

fully ripened bananas on the 16 days of storage were chosen for PE extraction. The SDS-PAGE diagrams of PE fractions with M.W. of 49.3-78.2 kDa indicated several isoforms as shown in Figure 1. The occurrence of PE isozymes was previously reported in hybrid aspen[4], flax[27], and tomato[28].

The molecular mass of banana PE in this study was higher than that of most plant PEs (32-35 kDa)[29]. Goldberg *et al.*[30] proposed that large molecule PEs, such as the neutral PE of mung bean hypocotyls (*Vigna radiata*) with a molecular mass of 45 kDa and kiwi PE with a molecular mass of 57 kDa[31], be glycoproteins. Presumably glycosylation occurs in banana too.

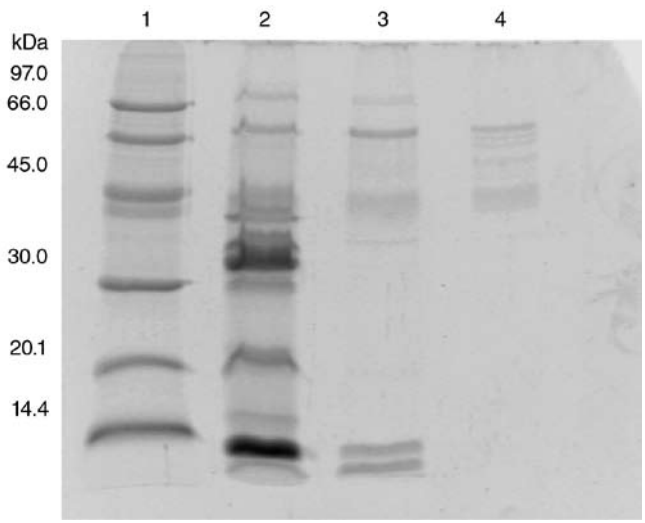

Figure 1. SDS-PAGE profiles of banana PE. (1) Molecular weight markers; (2) ammonium sulfate (30-70% saturation) purified; (3) CM-Sepharose CL-6B chromatography purified; (4) Sephacryl S-200 HR chromatography purified. Polypeptides on the gel were stained with Coomassie brilliant blue.

II. *Determination of PEI*

The determination of PEI in this study was made by traditional pH-stat and the proposed ELISA method. As shown in Figure 2, there are good linear responses with the activity of banana PEI in both of the pH-stat ($R^2 = 0.959$) and the ELISA ($R^2 = 0.945$) methods. The responses to PEI activities in kiwi ($R^2 = 0.935$) and jelly fig achenes ($R^2 = 0.983$) in PEI-ELISA method are also linear (Figure 3). The good correlation between PEI concentration and ELISA value and the successful determination of PEI in jelly fig (*Ficus awkeotsang* Makino) achene using anti-banana PE antibodies implied that PEI-ELISA method is suitable for PEI quantification. Our assumption was also proven that PE and PEI form a complex in a ratio of 1:1. The reported natural inhibitors of PE include the uncompetitive inhibitors sucrose[32], KI and Na-dodecyl sulfate[12-13], the competitive inhibitor polygalacturonic acid[33-34], and PEI. The addition of the competitive inhibitor jelly fig PEI caused an increase of K_M for PE-pectin reaction[16]. Therefore, to determine PEI by PEI-ELISA method that is based on the interaction between PEI and PE, is feasible even in the presence of other natural inhibitors of PE.

III. *Changes of PEI Activity in Postharvest Storage*

There was no significant difference ($p > 0.05$) in PEI activity within 16 days storage at 10°C. However, the activity dropped significantly ($p < 0.05$) after switching to room temperature (Figure 4). The activity also dropped significantly ($p < 0.05$) after day 10 during storage at room temperature from the beginning (Figure 5).

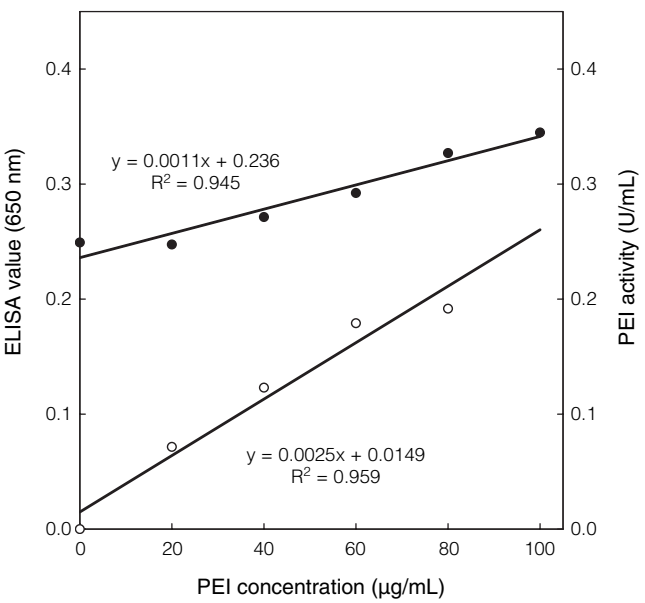

Figure 2. Determination of banana PEI by ELISA (●) and pH-stat (○) methods.

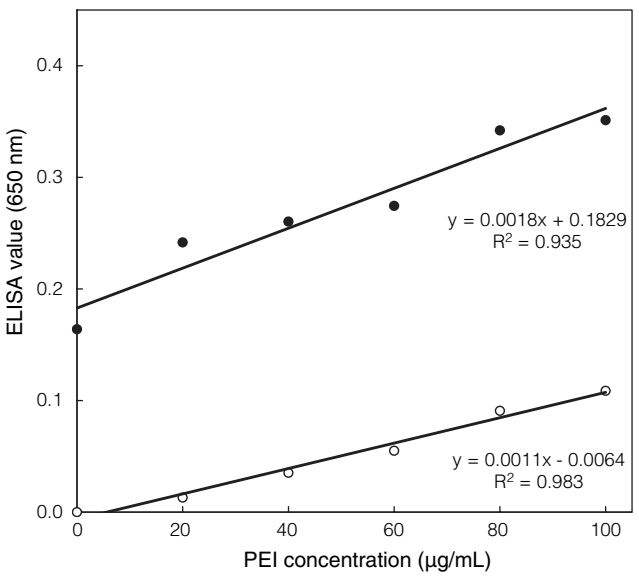

Figure 3. Determination of PEI from kiwi and jelly figachenes by ELISA method.

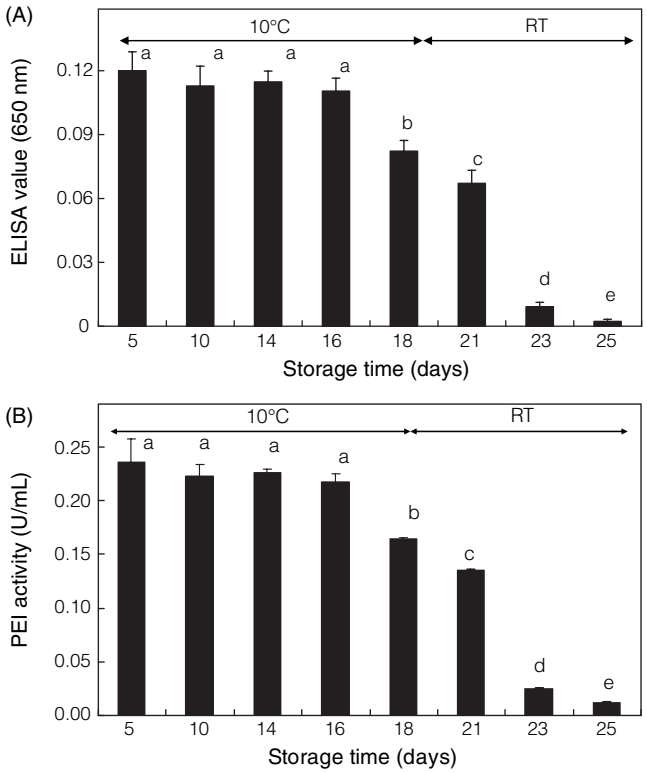

Figure 4. Changes in banana PEI analyzed by (A) ELISA and (B) pH-stat methods during storage at 10°C for 18 days and at room temperature for another 7 days. Data bearing different superscript letters were significantly different ($p < 0.05$).

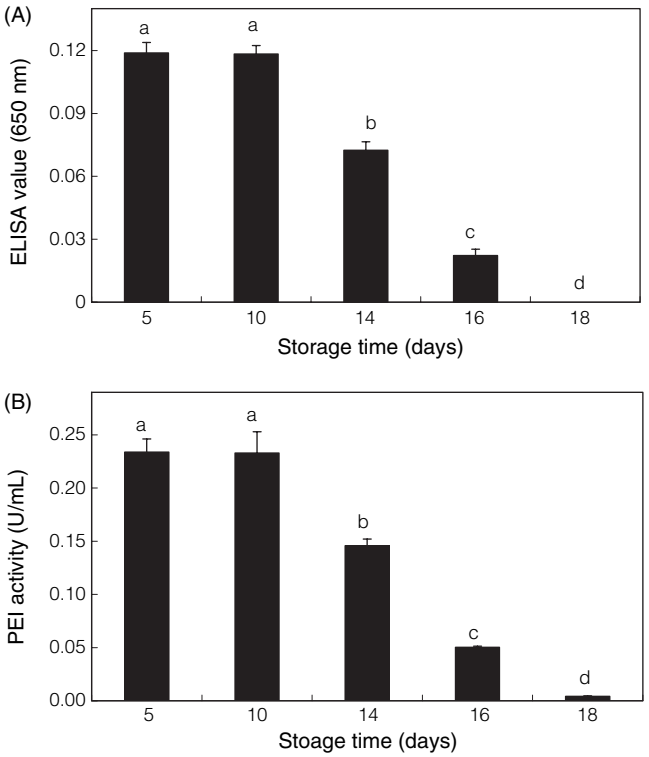

Figure 5. Changes in banana PEI analyzed by (A) ELISA and (B) pH-stat methods during storage at room temperature for 18 days. Data bearing different superscript letters were significantly different ($p < 0.05$).

No significant difference ($p > 0.05$) was found when comparing the corresponding data of banana PEI activity determined by pH-stat and PEI-ELISA methods in the above-mentioned storage tests.

Banana is highly susceptible to chilling injury[35]. Low temperature storage often results in pitting and discoloration of the peel and abnormal ripening of the pulp. Pulp softness, sweetness and flavor of modified atmosphere packed banana were found to be better than the fruit stored at 10°C for 12 or 18 days and then ripened at room temperature (28°C) for 4 days[36]. The puncture force of banana was decreased in the order of 10, 16, 22 and 28°C of storage[37]. Pathak and Sanwal[38] and Marín-Rodríguez *et al.*[39] proposed that PEI might play an important role in banana pulp softness caused by pectic enzymes and delay the ripening of banana fruits, especially at low temperature. Our experiments confirmed that the retardation in the ripening of banana pulp in low temperature storage is relevant to the maintenance of PEI activity.

CONCLUSIONS

ELISA is generally regarded as a precise analytical method with great specificity. The use of ELISA involving a polyclonal antibody against banana PE to quantify the PEI in jelly fig achenes, kiwi fruit and banana pulp is proven successful. PEI-ELISA method has the advantage to avoid the interference from the buffer components in the sample and the alkali added in the conventional pH-state PE inhibition assay. In the present study much more PEI is needed in the pH-state method than in the ELISA method. The ELISA method is more sensitive than the pH-state method, thus saving raw material and the effort in the preparation samples.

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