Change in Particle Size of Pectin Reacted with Pectinesterase Isozymes from Pea (*Pisum sativum* L.) Sprout

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Four pectinesterase (PE) isozymes were isolated by CM-Sepharose CL-6B chromatography from etiolated pea (*Pisum sativum* L.) sprouts and then reacted with citrus pectin (degree of esterification = 68%, 30−100 kDa) to observe the change in pectin particle size using a laser particle size analyzer. After incubation of a pectin−PE mixture (pH 6.5) at 30 °C for 4 h, PE 1 was observed to catalyze the transacylation reaction most remarkably, increasing the particle size from ∼50−70 to ∼250−350 nm, followed by PE 3, PE 2, and PE 4.

Keywords: *Pectinesterase isozyme; pea (Pisum sativum L.) sprout; transacylation reaction; de-esterification reaction; laser particle size analysis*

INTRODUCTION

Pectinesterases (pectin pectyl-hydrolase, EC 3.1.1.11) (PEs) have been found in all species of higher plants and are also produced by a number of plant pathogenic fungi and bacteria (*1*). Plant PEs participate in the conversion of protopectin to soluble pectin and pectate by catalyzing the de-esterification of pectin, and they are involved in processes of fruit maturation.

Because PE is a hydrolase, according to the doublee-displacement mechanism, it reacts with the C_6 carboxyl groups of D-galacturonic acids to form unstable complexes by releasing methanol and then transfers the C_6 carboxyl groups to an adequate acceptor (such as a water molecule) to complete the de-esterification reaction. However, the hydroxyl groups in the pectin molecules are occasionally acceptors of such a reaction, thus leading to the formation of high molecular weight pectins (*2*).

Recently, a marked increase in the molecular weight of pectin reacted (pH 6.5, 45 °C, 2 h) with PE from jelly fig (*Ficus awkeotsang* Makino) achenes was observed by gel permeation chromatography using a 3 M urea/0.2 M NaCl/0.05 M acetate buffer (pH 5.5) as eluent (*3*). In addition, non-methyl ester linkages, the difference between the total ester linkage (*4, 5*) and the methyl ester linkage (*6*), increased by approximately 40% during jelly fig pectin gelling (*7*) and 50% before and after precooking (50−70 °C) and cooking treatment (100 °C) of pea sprouts (*2*). This revealed the esterification reaction between C_6 carboxyl groups and hydroxyl groups in the presence of PE (*2, 3, 7*).

For further investigation of the transacylation reaction, in the current study isolated PE isozymes from pea sprouts were reacted with citrus pectin, and the change in particle size of reacted pectin was determined by using a laser light-scattering method. Finally, the relationship between these two reactions was discussed.

MATERIALS AND METHODS

Materials. Citrus pectin (lot 79F0188, Sigma Chemical Co., St. Louis, MO) of 68% degree of esterification (DE) and 980 kDa (average molecular weight) was used for transacylation reaction after removal of ions according to the method described by Jiang et al. (*7*). In brief, pectin was first dissolved in 5 volumes of 0.4% sodium hexametaphosphate (Sigma Chemical Co.) and allowed to rest for 24 h, followed by dialysis against flowing deionized water for 48 h to remove the ions possibly present in the pectin sample. The pectin precipitates obtained after filtration through a No. 2 Whatman filter paper were rinsed with equal volumes of 2-propanol and an adequate volume of acetone to prepare the pectin powder.

Other chemicals were of analytical grade. Pea (*Pisum sativum* L.) beans were purchased from a local supermarket.

Preparation of PE Isozymes. Pea beans were dipped in tap water for 10 min in a beaker and then incubated at room temperature (28 ± 2 °C) in darkness for 9 days in a sprouting incubator (Homelife, Chan-Lo Co., Taipei, Taiwan) equipped with an automatic water-spray system. Tap water was sprayed on the beans and sprouts every 5 h during the incubation period. Endosperms of the harvested sprouts were removed prior to use.

An adequate amount (50 g) of thusly harvested etiolated pea sprouts was blended (Osterizer, Sunbeam-Oster Household Products, Schaumburg, IL) for 2 min with 3 volumes of cold (4 °C) deionized water (Milli-Q system, Millipore, Osaka, Japan), and the residues were collected by filtration with six layers of cheesecloth. After being washed twice, the collected residues were subsequently blended with 3 volumes of 0.01 M phosphate buffer solution (pH 8.0) for 2 min, followed by filtration with six layers of cheesecloth. The residues thus obtained were finally blended with 2 volumes of 0.75 M NaCl for 2 min to extract the PE enzymes. Supernatants (crude PE enzyme solution) collected by centrifugation (16300*g*, 4 °C, 30 min) were further fractionated according to the following procedures.

Isozymes in crude PE enzyme solution were preliminarily fractionated by 100% ammonium sulfate. The precipitates

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collected by centrifugation (10000*g*, 4 °C, 30 min) were dialyzed against 100 volumes of 0.01 M phosphate buffer (pH 8.0) at 4 °C for 12 h, followed by CM-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) ion-exchange chromatography. Experimental conditions for CM-Sepharose CL-6B ion-exchange chromatography were as follows: column size, 2.6 × 28 cm; elution buffer, 0−0.5 M NaCl/0.01 M phosphate buffer (pH 8.0); fractionation, 4.0 mL/tube; flow rate, 40 mL/h. Fractions corresponding to each PE isozyme were pooled and dialyzed against 0.01 M phosphate buffer (pH 8.0), followed by concentration with a membrane concentrator (Amicon Co.; MWCO = 10 kDa). The PE concentrate thus obtained was well mixed with 50% glycerol to 100 units/mL and then refrigerated (−20 °C) until use.

Extraction and isolation of enzyme were thoroughly conducted in a chilled room (4 ± 1 °C). PE isozymes obtained in the present study were PEs 1, 2, 3, and 4, in the sequence of their elution order of the chromatogram of CM-Sepharose CL-6B ion-exchange chromatography.

Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE (12.5% acrylamide gel) was performed according to the method of Laemmli (*8*) with the pooled and dialyzed (against distilled water) eluate of CM-Sepharose CL-6B ion-exchange chromatography.

Determination of PE Activity. PE activity was determined according to the method described by Lee and MacMillan (*9*) with minor modifications. One milliliter of enzyme solution was added to 15 mL of 0.1 M NaCl/0.5% citrus pectin solution (30 °C) with the pH adjusted to 6.0 immediately before assay. The activity of PE was measured by titrating (pH M83 Autocal pH meter, TTT 80 titrator, ABU80 autoburet, Radiometer Copenhagen Co., 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 for the reaction solution at 30 °C (in a water bath) were recorded within a reaction time of 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. Triplicate samples each were analyzed twice.

Transacylation Reaction. The pH value of the 0.4 M NaCl/1.0% citrus pectin solution was adjusted to ∼6.5 by adequate addition of 0.1 N NaOH solution. Then 6 mL of pectin solution was mixed well with an equal volume of 0.2 M phosphate buffer (pH 6.5). After centrifugation (16300*g*, 10 min), the obtained supernatants were filtered through a 0.45 μm membrane. Ten milliliters of the pectin solution thusly obtained was mixed well with 0.1 mL of PE 1, 2, 3, or 4 isozyme (100 units/mL). The PE−pectin mixtures were incubated at 30 °C in a water bath for 4, 8, or 16 h with slight stirring by a magnetic stirrer, followed by thermal treatment in boiling water for 3 min to stop the reaction. The particle sizes of the thusly obtained pectin solutions in a well-washed and dried cuvette were then determined by a laser particle size analyzer (3 nm−5 μm) (model PAR III, Photal Otsuka Electronics Co. Ltd., Tokyo, Japan) with a dynamic light-scattering method.

In comparison, PE 1 inactivated in 5 mM I_2/50 mM KI solution previously for 10 min (*10*) was added to pectin solution (control).

Determination of Pectin. Pectin content was determined using the method described by Blumenkrantz and Asboe-Hansen (*11*). An adequate volume (0.5 mL) of pectin solution was mixed well with 3 mL of 0.0125 M sodium tetraborate solution (in concentrated sulfuric acid) and then heated in a boiling water bath for 5 min. After cooling in an iced bath, the reaction mixture was uniformly mixed with 0.05 mL of 0.15% *m*-phenylphenol/0.5% NaOH solution and allowed to rest for 5 min. Color developed at 520 nm was recorded with a spectrophotometer (model 7800UV/VIS, Jasco). The standard solution was constructed using different levels (0−100 μg/mL) of D-galacturonic acid (Sigma) to compute the pectin content in samples. Triplicate samples each were analyzed twice.

Determination of Protein. Protein concentrations in solutions containing PE were assayed according to the Bradford (*12*) method using Bio-Rad protein assay dye reagent. Bovine serum albumin (0.2−1.4 mg/mL) was used to construct the standard curve. Triplicate samples each were analyzed twice.

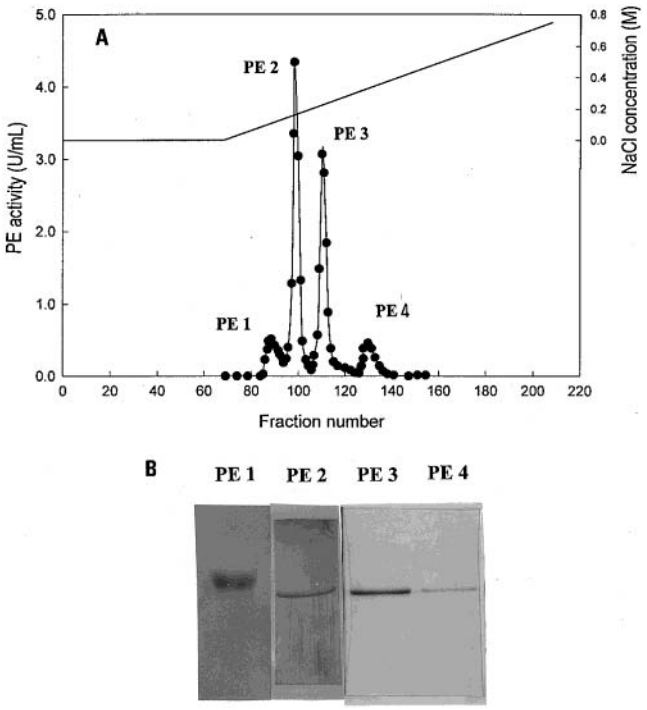

Figure 1. Elution profile (A) of 100% ammonium sulfate precipitates from etiolated pea sprouts on CM-Sepharose CL-6B ion-exchange chromatography, and SDS-PAGE (12.5% acrylamide gel) (B) of the isolated PE isozymes. Etiolated pea sprouts were harvested after 9 days of incubation in darkness at room temperature. Experimental conditions for ion-exchange chromatography: column, 2.6 × 28 cm; elution buffer, 0−0.5 M NaCl/0.01 M phosphate buffer (pH 8.0); flow rate, 40 mL/h; fractionation, 4 mL/tube.

RESULTS AND DISCUSSION

For the ease of the observation of the change in particle size of pectin in the present study, pectin of ∼980 kDa from a citrus source was used. The molecular weight of pectin was determined by Fractogel HW-65 (F) (E. Merck, Darmstadt, Germany) gel permeation chromatography (data not shown) using Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) and Shodex Standard P-82 kit (Pullulan, 78.8−0.59 × 10^4 kDa) (Showa Denko K.K., Tokyo, Japan) to construct the standard curve.

Isolation of PE Isozymes. Endosperms were removed from the etiolated pea sprouts, and the 100% ammonium sulfate precipitates of the supernatant from sprout homogenate were applied to the CM-Sepharose CL-6B ion-exchange chromatography. Figure 1 A represents the isolated PE isozymes as eluted by increasing NaCl levels (0−0.5 M) in the 0.01 M phosphate buffer (pH 8.0). PE 1 was eluted first (fraction 85) by 0.13 M NaCl, followed by PE 2 (fraction 98) by 0.18 M NaCl, PE 3 (fraction 109) by 0.24 M NaCl, and finally by PE 4 (fraction 127) by 0.34 M NaCl. All of the collected isozymes were applied to SDS-PAGE after dialysis against distilled water, and the results were as shown in Figure 1B. A single protein band was observed in the gel of each isozyme, revealing the PE isozymes are all single peptides. The thusly obtained PE isozymes were used in the following PE−pectin interaction.

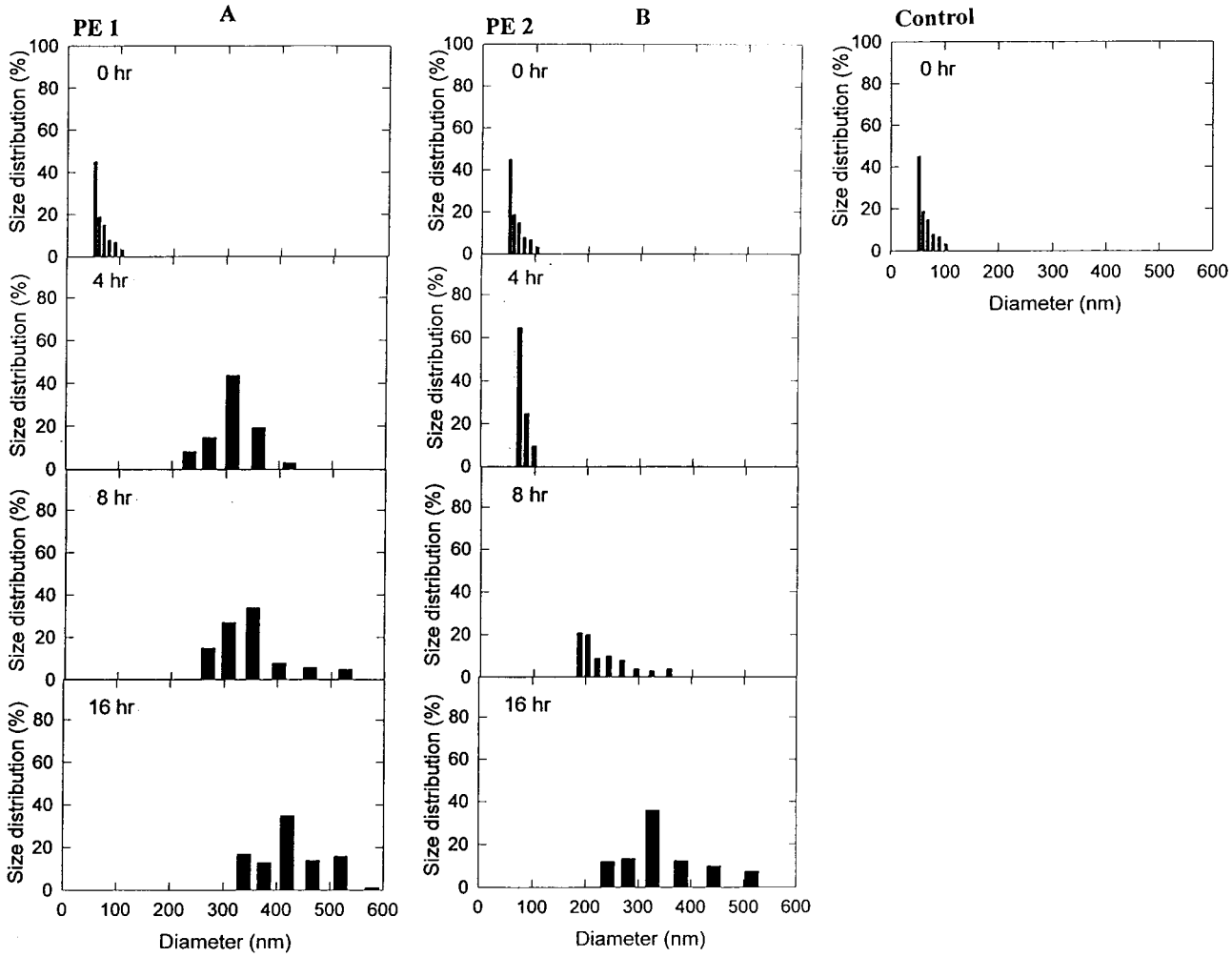

Figure 2. Changes in particle sizes of citrus pectins reacted with PEs 1 (A) and 2 (B) isolated from etiolated pea sprouts for up to 16 h. Ten milliliters of 1% pectin solution was mixed with 0.1 mL of PE (100 units/mL), and the mixtures were incubated at 30 °C, pH 6.6, for 4, 8, and 16 h prior to laser particle analysis. PE 1 denatured by 5 mM I_2/50 mM KI was used as control.

Change in Particle Size of Pectin. Figure 2A represents the changes in particle sizes of pectin reacted with PE 1. The particle size of citrus pectin used as starting material was mainly distributed between 50 and 70 nm (diameter), as detected by a laser light-scattering particle size analyzer. However, after reaction with PE 1 for 4 h at pH 6.5, the distribution of the diameters of pectin particle sizes increased to mainly between 250 and 350 nm, suggesting a remarkable increase in pectin molecular weight resulting from the transacylation reaction between interpectin molecules. However, the I_2- and KI-inactivated PE 1 (control) did not show any change in the particle size of pectin (Figure 2) during the 4 h of incubation. More remarkable increases in pectin particle size were observed when the reaction time was prolonged to 8 h (mainly between 300 and 350 nm in diameter) and to 16 h (mainly 425 nm in diameter). Beri et al. (*13*) reported the characterization such as molecular weight and radius of gyration data of nine chitosan samples, differing in the degree of acetylation and molecular weight, via coupled size exclusion chromatography and a multiple-angle laser light-scattering technique. Hourdet and Muller (*14*) determined the conformation and molecular size of high galacturonic acid in pectin chains using a light-scattering method.

Previously, an increase in the molecular weight of pectin reacted with PE from jelly fig (*Ficus awkeotsang* Makino) achenes was observed when a citrus pectin−PE mixture was incubated at 45 °C in 0.2 M NaCl for 20 min. In addition, prolonging the incubation time of the pectin−PE mixture to from 2 and 4 h also enhanced the molecular weight of pectin (*3*). However, such an increase in pectin molecular weight was completely stopped when the pectin−PE mixture was reacted with 0.1 M Na_2CO_3 (*3*). Hou and Chang (*2*) also reported an increase in the molecular weight of citrus pectin as a result of the transacylation reaction catalyzed by PE from pea sprouts, as determined by gel permeation chromatography using acetate buffer (pH 5.5) containing 3 M urea as eluent. It was also noteworthy that the transacylation reaction of pectin was relevant to the firming of vegetable tissues during precooking (50−70 °C) and cooking treatment (100 °C). Furthermore, during the preparation of jelly curd, non-methyl ester linkages increased from 0.050 to 0.071 μmol of $CH_3CONHCO$/mg of pectin when jelly fig pectin was extracted with tap water for 1 h and rested for another 1 h. This revealed the formation of an esterification reaction between C_6 carboxyl groups and hydroxyl groups of pectin molecules in the presence of PE (*7*). Similar results were also observed by Hou and Chang (*2*) during the precooking and cooking treatment of pea sprouts.

Compared with PE 1, PE 2 (Figure 2B) and PE 3 (Figure 3A) exhibited less remarkable transacylation

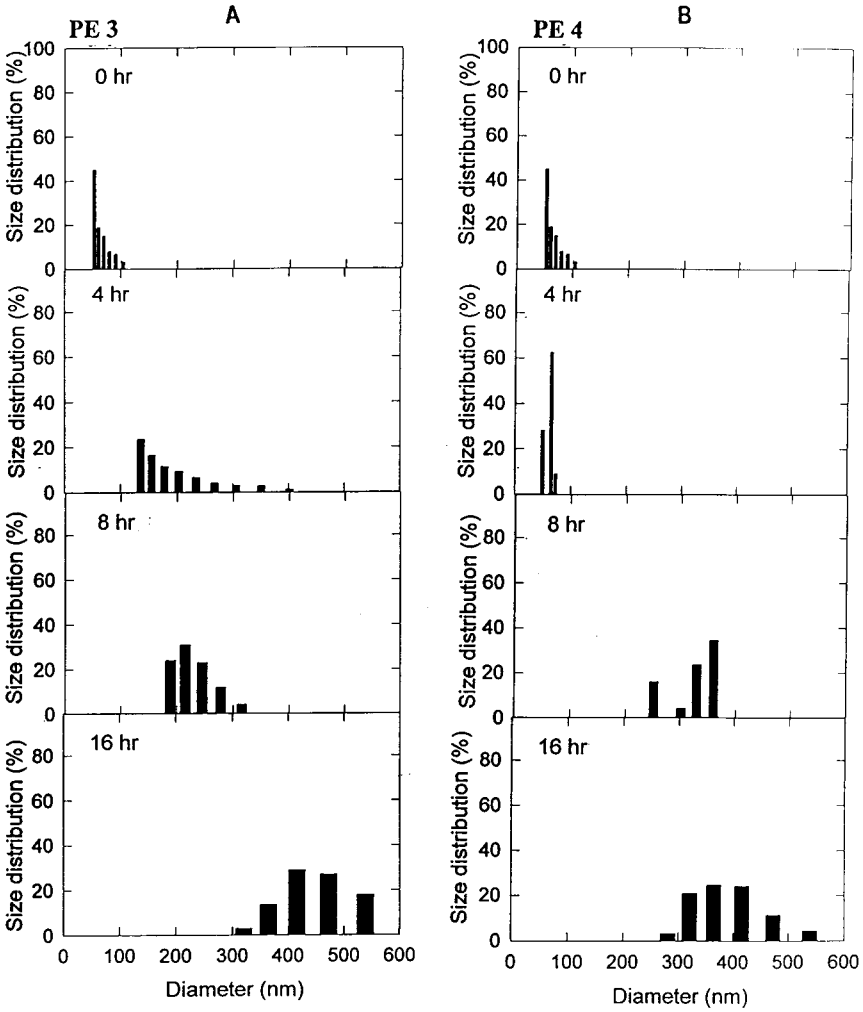

Figure 3. Changes in particle sizes of citrus pectins reacted with PEs 3 (A) and 4 (B) isolated from etiolated pea sprouts for up to 16 h. Experimental conditions were the same as in Figure 2.

reactions, mainly increasing the diameter of pectin particles to about 70−80 and 140−200 nm, respectively, after 4 h of interaction. At 16 h, diameters of pectin particle sizes in pectin−PE mixtures had increased to mainly 340 and 420−480 nm as the result of the transacylation induced by PE 2 and PE 3, respectively. The effect of PE 4 (Figure 3B) on the pectin particle size was similar to that of PE 3, increasing the particle size of pectin to between 320 and 420 nm in diameter after 16 h of interaction. Therefore, the particle size increasing effect by PE isozymes is apparently in the order of PE 1 > PE 3 > PE 2 > PE 4. PE 4 also showed a less significant increase in pectin molecular weight when it was incubated with pectin at 60 °C for 20 min, as determined by gel permeation chromatography (*2*).

In the present study, pea sprout PE exhibited a remarkable transacylation reaction in 0.2 M NaCl/0.1 M phosphate (pH 6.5), which was close to the optimal condition (0.1 M NaCl, pH 6.0) of de-esterification reaction by pea PE (*15*). Similar results were also observed by Jiang et al. (*3*), where the optimal conditions for the de-esterification reaction (0.1−0.65 M NaCl, pH 6.5) and for the transacylation reaction (0.2 M NaCl, pH 6.5) of citrus pectin by jelly fig PE were similar. Therefore, an increase in pectin molecular weight may occur while a de-esterification reaction proceeds. A marked increase in the molecular weight of pectin reacted with PE for 20 min in 0.05 M NaCl/0.1 M phosphate (pH 6.5) was observed by a Fractogel TSK HW-65 (S) gel permeation chromatography (*2*, *3*). Thus, it is possible that pectin is converted into low-methoxyl pectin in the presence of PE and the exposed C_6 carboxyl groups in D-galacturonic acid then react with hydroxyl groups of interpectin molecules to advance the transacylation reaction.

Conclusions. Particle sizes of pectins reacted with PE isozymes were determined by a laser light-scattering particle analyzer, and PE 1 showed the most remarkable increasing effect on pectin particle size. Compared to the gel permeation chromatographic method, laser particle size analysis appeared to be much more convenient. Excessive increase in pectin molecular weight, resulting from PE transacylation reaction, may lead to clogging of the column and the resultant incorrect observation on change in pectin molecular weight by conventional size exclusion methods.

The increase in the particle size of pectin in the presence of PE has been proposed to be a result of the transacylation reaction between interpectin molecules (*2*, *3*). However, such a reaction may also occur between intrapectin molecules in the presence of PE.

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