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# Transacylation and De-esterification Reactions of Pectin As Catalyzed by Pectinesterases from Tomato and Citrus

JEAN-YU HWANG,† CHING-WEN LEE,‡ MING-CHANG WU,§ AND HUNG-MIN CHANG*,‡

Department of Food Nutrition, Chung-Hwa Institute of Technology, Tainan, Taiwan, Graduate Institute of Food Science and Technology, National Taiwan University, Taipei 10617, Taiwan, and Department of Food Science, National Pingtung University of Science and Technology, Pingtung 91207, Taiwan

The optimal conditions for the de-esterification reaction of tomato pectinesterase (PE) and citrus PE was 0.1−0.2 M NaCl and at pH 7.5−8.5, 65 °C, almost identical to those for the transacylation reaction as observed by turbidity (absorbance at 400 nm) change. Among the PEs tested, pea pod PE presented the most remarkable catalysis on the transacylation reaction, and 1.5% pectin solution was determined to be suitable for this reaction. Low methoxy pectin with a DE (degree of esterification) of 31% displayed a slow turbidity increase, revealing that the extent of DE was influential on the transacylation. Besides citrus pectin, apple pectin was also proved to progress transacylation reaction by PEs from tomato and citrus sources as apparently observed by turbidity method.

**KEYWORDS: De-esterification reaction; transacylation reaction; tomato PE; citrus PE; turbidity test**

## INTRODUCTION

Pectinesterases (pectin pectyl-hydrolase, EC 3.1.1.11) (PEs) are present in all species of higher plants and are also produced by a number of plant pathogenic fungi and bacteria (*1*). Plant PEs convert protopectin to soluble pectin and pectate by catalyzing the de-esterification of pectin. Decreasing levels of esterification are crucial to the fruit maturation process.

PE, as a hydrolase, according to the double displacement mechanism, reacts with the $C_6$ carboxyl groups of D-galacturonic acids to form unstable complexes by releasing methanol. Then, the $C_6$ acyl groups are transferred to adequate acceptors, such as water molecules, to complete the de-esterification reaction. However, the hydroxyl groups of pectin molecules are occasionally acceptors of such a reaction, and therefore form high molecular weight pectins (*2*−*4*). Citrus pectins (MW = 980 kDa) with an averaged degree of esterification (DE) of 68% were increased in particle size (diameter) from about 100 to 400 nm after incubation with pea sprout PE isozymes at 30 °C, pH 6.6 for 16 h, as determined by a laser light-scattering analyzer (*4*). Similarly, a marked increase in the molecular weight of citrus pectin was observed by a gel permeation chromatography when pectin was reacted (pH 6.5, 45 °C, 2 h) with PE from jelly fig (*Ficus awkeotsang* Makino) achenes (*3*). Above findings reveal the esterification reaction between $C_6$ carboxyl groups and hydroxyl groups in pectin molecules in the presence of PE (*2*−*4*). Therefore, de-esterification reaction and transacylation reaction of PE are considered to proceed simultaneously.

Some proteases including trypsin (*5*), subtilisin (*6*), and papain (*7*) catalyze the transacylation reactions (Plastein reaction) while hydrolyzing proteins. Instead of transferring the acyl groups of amino acids to water molecules to fulfill the hydrolysis process, those proteases transfer acyl groups to the hydroxyl groups of peptides at weak acidic pHs (pH 4−6). Thus, within this pH range, new polypeptide compounds were formed as a result of the increase in molecular weights of reaction mixtures. Protein modification by means of Plastein reaction is available in preparing hydrophilic proteins.

It is noteworthy that the transacylation reaction condition of citrus pectin with PEs from pea sprout or from jelly fig achenes was close to their respective optimal de-esterification reaction condition (*3*, *4*). In the present study, to understand the transacylation reactions of PEs from tomato and citrus, the vast fruit sources for food processing, turbidity observation on pectin-PE mixture versus reaction time was conducted using pea pod PE as positive control. Then, the conditions for both de-esterification and transacylation conditions of PEs were optimized and compared. Finally, citrus pectins with various DEs and pectins from different sources were tested.

## MATERIALS AND METHODS

**Materials.** Citrus pectins (DE = 31, 67, and 89%) used were products of Sigma Chemical Co. (St. Louis, MO), whereas apple pectin (DE = 73%) was purchased from Fluka Co. (Buchs, Switzerland). Pectins used for transacylation reaction were treated to remove ions, especially divalent cations, possibly presented in pectin samples

* To whom correspondence should be addressed. Tel.: +886-2-23630231, ext. 2776. Fax: +886-2-23620849. E-mail: changhm@ccms.ntu.edu.tw.
† Chung-Hwa Institute of Technology.
‡ National Taiwan University.
§ National Pingtung University of Science and Technology.

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according to the method described by Jiang et al. (*4*). In brief, pectin was first dissolved in 5 volumes (w/v) of 0.4% Na-hexametaphosphate (Sigma) 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 2-propanol and acetone to prepare the pectin powder. The pectin powder thus obtained was used to prepare the substrate solution for the subsequent transacylation reaction described below. Pectins with DE of 31 and 89% were used to compare the effect of DE on the transacylation reaction.

**Preparation of PEs from Pea Pod, Tomato, and Citrus.** Pea (*Pisum sativum* L.) pod PE was prepared according to the method described by Jiang et al., (*4*). In brief, pea pod was homogenized (in a blender) with cold (4 °C) deionized water and 10 mM phosphate buffer (pH 8.0) to remove the soluble portions, and then the residues were blended with 0.3 M NaCl to extract crude PE. Subsequently, ammonium sulfate precipitation (60−80% saturation) and CM-Sepharose CL-6B ion-exchange chromatography (eluent, 0−0.5 M NaCl/100 mM phosphate buffer, pH 8.0) were conducted to collect the purified pea pod PE *(4)*.

Extraction of tomato PE was conducted following the methods described by Pressey and Avants (*8*). Edible portions of fruits were homogenized with 0.25 M NaCl/0.1 M phosphate buffer (pH 8.0) at 4 °C overnight. Supernatants by centrifugation (10000*g*, 1 h, 4 °C) were added with ammonium sulfate to collect the precipitates of 0−80% saturation for CM-Sepharose CL-6B ion-exchange chromatography (eluent, 0−1 M NaCl/0.1 M phosphate buffer, pH 8.0).

Citrus PE was extracted from edible portions of Sunkist from a local market with 1 M NaCl/0.3 M phosphate buffer for 2 h at 4 °C (*9*). Supernatant (crude citrus PE) by centrifugation (12000*g*, 30 min, 4 °C) was treated with ammonium sulfate to collect the precipitates of 30−80% saturation, which were further purified by a CM-Sepharose CL-6B ion-exchange column, as described for the purification of tomato PE, to collect the fractions with PE activity.

After dialysis against 0.1M NaCl/0.1 M phosphate buffer (pH 6.5) and membrane concentration (Amicon Co., MWCO = 10 kDa), PE solutions thus obtained were mixed with 0.1M NaCl/0.1 M phosphate buffer (pH 6.5) to the desired activity for the following reactions with pectin.

**Optimization of De-esterification Reactions of PEs.** Pectin solutions (0.5% pectin/0.1 M NaCl) with various pH values (pH 3.5−8.5), adjusted with 0.1 N NaOH, were previously incubated at room temperature (22−25 °C) for 5 min to react with PEs (2 units/mL mixture) from tomato and citrus sources to determine the pH effect on the de-esterification reaction. Substrate solution (0.5% pectin/0.1 M NaCl) at pH 6.5 were reacted with PE (2 units/mL mixture) at temperatures ranging from 25° to 85 °C in a water bath for 5 min to determine the optimal temperature. On the other hand, to determine the effect of NaCl on PE activity, NaCl content in substrate solution at pH 6.5 was adjusted to 0−1.0 M to react with tomato PE and citrus PE (2 units/mL mixture) at room temperature.

**Turbidity Observations on Transacylation Reaction of PEs.** Various levels (0.5, 0.75, 1.0, 1.25, 1.5, 2.0, and 2.5%) of citrus pectin (DE = 68%) in 0.1 M NaCl/0.1 M phosphate buffer (pH 6.5) were prepared. To 2.0 mL of those pectin solutions was added 0.1 mL of PE (2 units/mL mixture) from pea pod, tomato, or citrus source to determine the pectin level on the reaction. The pectin-PE mixtures in cuvettes were incubated at room temperature, and the turbidity change (absorbance at 400 nm) during the incubation for up to 15 h was monitored by a spectrophotometer (UV/VIS spectrophotometer, Helios Alpha, Spectronic Unican Co. Ltd., Great Britan).

A similar procedure was conducted to determine the pH effect on the reaction, except that 1.5% pectin solutions at pH 5.5, 6.5, and 7.5 were used. Enzyme activity in 1.5% pectin−PE mixtures was adjusted to 0.5, 1.0, 1.5, 2, and 2.5 units/mL mixture to observe the effect of PE activity on the transacylation reaction. In addition, 1.5% pectin-PE mixtures (2 units/mL mixture) containing 0, 0.25, 0.4, 0.55, and 0.7 M NaCl were incubated for up to 45 min to detect the influence of NaCl level on the reaction. Pectins with DE of 31, 67, and 87% were dissolved (1.5%, w/v) in 20 mM EDTA/0.1 M NaCl/0.1 M phosphate buffer (pH 6.5) to prepare pectin solutions to determine the effect of pectin DE on the transacylation reaction. Citrus pectin and apple pectin (1.5%) were incubated with each of three PEs for up to 45 min to observe the effect of PEs on the pectin source. An enzyme solution previously heated in boiling water for 5 min was used as a blank. The experiment was repeated three times with similar results.

**PE Activity Assay.** PE activity was determined according to the method described by Lee and MacMillan (*10*) with minor modifications. One mL of PE solution was added to 15 mL of 0.1 M NaCl/0.5% citrus pectin solution (substrate solution) (25 °C) with the pH adjusted to 6.5 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 (mL) of 0.01 N NaOH consumed to maintain a pH of 6.0 of the reaction solution at 25 °C (in a water bath) were recorded within 5 min. An activity unit represents 1 $\mu$eq of the free carboxyl groups produced by the PE hydrolytic activity on the pectin substrate per min at 25 °C. An enzyme solution previously heated in boiling water for 5 min was used as a blank. Triplicate samples each were analyzed twice. Deionized water (Mili-Q system, Millipore, Tokyo, Japan) was used to prepare the substrate and enzyme solutions.

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

**Determination of Protein.** Protein content in solutions containing PE was determined by the Bradford (*12*) method using the Bio-Rad protein assay dye reagent. Bovine serum albumen (0.2−1.4 mg/mL) was used to construct the standard curve ($r^2 = 0.989$). Triplicate samples were each analyzed twice.

## RESULTS AND DISCUSSION

Pea pod PE purified by ion-exchange chromatography presented 4 isozymes (*4*). Tomato PE was extracted and purified by a CM-Sepharose CL-6B column to obtain an enzyme solution (78.4 units/mg protein) (data not shown) containing two PE isozymes (*13*, *14*). On the other hand, citrus PE extracted (*9*) and purified by the same chromatography presented an enzyme solution with a specific activity of 56.0 units/mg protein (data not shown). Two isozymes were also observed from the ion-exchange chromatograms (data not shown), as were indicated by Versteeg et al. (*15*), Koner et al. (*16*), and Rillo et al. (*17*) using the different chromatographic systems. All the fractions exhibiting PE activity were pooled for the pectin−PE reaction.

**Optimization of De-esterification Reactions of PEs. Figure 1** presents the optimal de-esterification reactions of PEs from tomato and citrus sources. Tomato PE activity increased with the increasing pH (pH 2.5−8.5) and displayed an optimal pH at about 7.5−8.5. This result is similar to those reported by Nakagawa et al. (*18*), Pressey and Avants (*13*), and Warrilow and George Jones (*19*). Although tomato PE displayed optimal pH values at weak alkaline, following PE experiments were conducted at weak acidic pH (pH 6.5) due to the instability of pectin at alkaline pH values (*10*).

Maximal activity of tomato PE was determined at about 65 °C (**Figure 1**), as reported by Broeck et al. (*20*). Higher temperatures induced the protein denaturation as a result of the unfolding of the tertiary structure of PE (*21*).

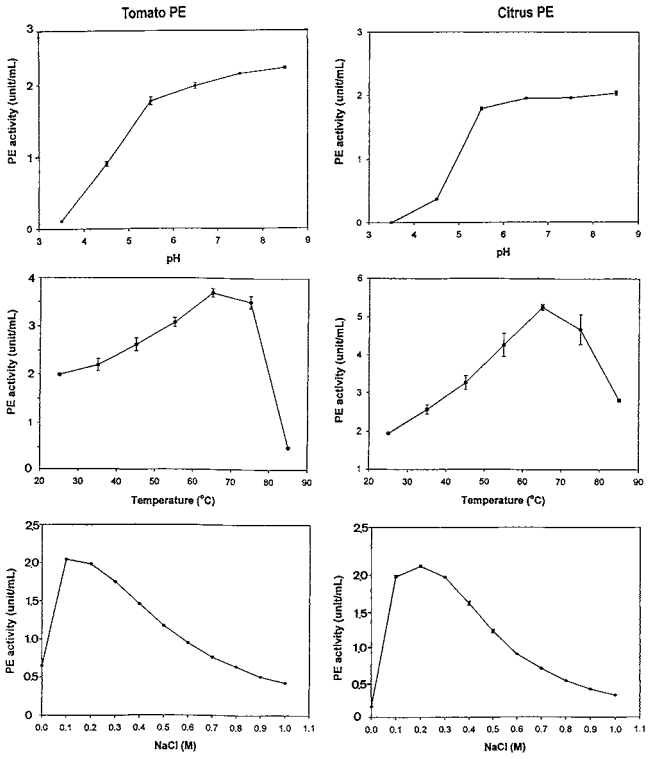


**Figure 1.** Optimization of tomato PE and citrus PE for de-esterification reactions. A 0.5% citrus pectin (DE = 67%)/0.1 M NaCl solution was reacted with PE (2 units/mL) under different pH, temperature, and NaCl conditions. The values represent means ± SE ($n$ = 3).

Warrilow and George Jones (*19*) pointed out that NaCl level was crucial to tomato PE activity. In **Figure 1**, tomato PE activity climbed sharply to reach 2 units/mL when NaCl increased from 0 to 0.1 M and then declined slowly to about 0.5 units/mL in the increased NaCl level (1.0 M NaCl). Therefore, on the basis of the above researches, optimal de-esterification conditions for tomato PE were considered to be 65 °C, pH 7−8, 0.1−0.2 M NaCl.

As for citrus PE, maximal activity was determined at pH values between 6.5 and 8.5, as indicated by Koner et al. (*16*) and Rillo et al. (*17*). Temperature change was also influential to citrus PE activity (*22*, *23*). Maximal activity of citrus PE was observed at 65 °C, close to that (60 °C) reported by Koner et al. (*16*). NaCl level in substrate solution (pH 6.5) affected the ionic interactions between the positively charged citrus PE (isoelectric point = 8.65) and the negatively charged pectin molecules. Therefore, NaCl was decisive to the PE activity. As it can be seen in **Figure 1**, 0.1−0.3 M NaCl was the optimal NaCl level for citrus PE. Thus, we concluded that the optimal conditions for citrus PE was 65 °C, pH 6.5−8.5, and 0.1−0.3 M NaCl.

**Turbidity Observations on Transacylation Reaction of PEs.** For convenience of the investigations on the transacylation reaction induced by PEs, turbidity method was adopted to observe the continuous progress of the reaction during the incubation for up to 15 h.

First, the effect of pectin level on the transacylation reaction of PEs from tomato and citrus sources was investigated, using pea pod PE as positive control enzyme (**Figure 2**). It was apparent that the increase in turbidity was dependent on the level of pectin and source of PE. For pea pod PE, turbidity change began at about 200 min and reached a maximal value of 1.6 (absorbance at 400 nm) when 1.5% citrus pectin-pea pod PE reaction mixture (2 units/mL mixture) was incubated at room

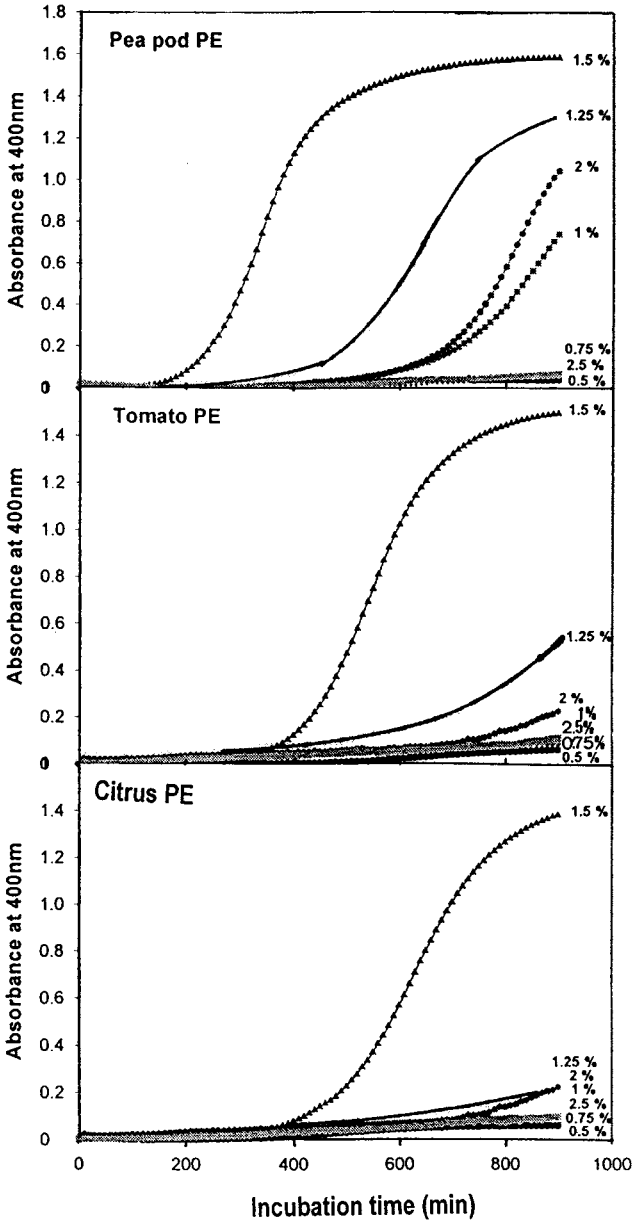


**Figure 2.** Changes in turbidity of citrus pectin (DE = 67%) at different levels (0.5–2.5%) reacted with various PEs (2 units/mL) in 0.1 M NaCl/0.1 M phosphate buffer (pH 6.5) for up to 15 h. The experiment was repeated three times with similar results.

temperature for about 700 min. High pectin level (2 and 2.5 %) was apparently unfavorable for the reaction, suggesting that high viscosity of pectin solution limited the pectin-PE reaction. The 1.5% pectin solution exhibited the most remarkable turbidity increase during the incubation, followed by 1.25, 2, and 1% pectin solutions. Solutions containing 0.75 and 0.5% pectin did not showed any change in turbidity, revealing low pectin solution was unfavorable for the transacylation reaction.

However, by means of gel permeation chromatography, Jiang et al. (*3*) observed the apparent molecular weight increase in 0.5% citrus pectin-jelly fig PE (2.5 units/mL mixture) solution when incubation was conducted at pH 6.5, 45 °C for 2 h. In addition, incubation (pH 6.5, 30 °C) of PE isozyme from pea sprout with citrus pectin for 4 and 16 h increased the pectin particle size from ∼50−70 to ∼250−350 nm and to 400 nm, respectively *(4)*, as determined by laser particle size analyzer. Therefore, increase in turbidity of pectin-PE mixture was due to the sufficient aggregations of high molecular weight-pectin

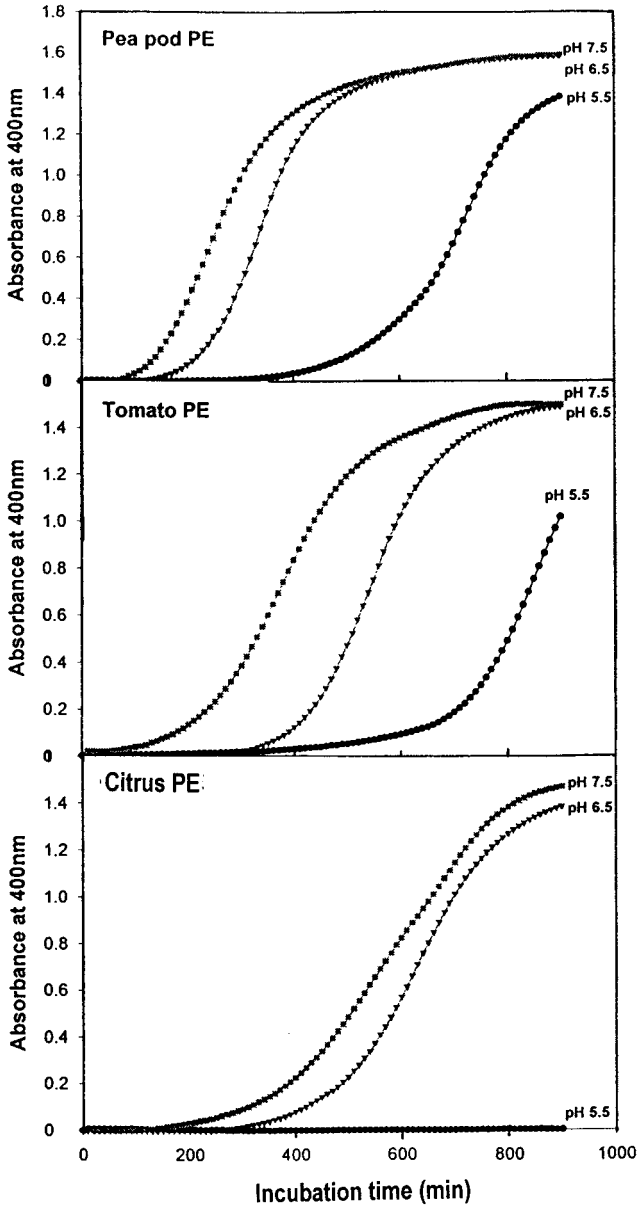


**Figure 3.** Changes in turbidity of 1.5% citrus pectin (DE = 67%)-PE mixtures (2 units/mL) in 0.1 M NaCl at pH 5.5, 6.5, and 7.5, and at room temperature for up to 15 h. The experiment was repeated three times with similar results.

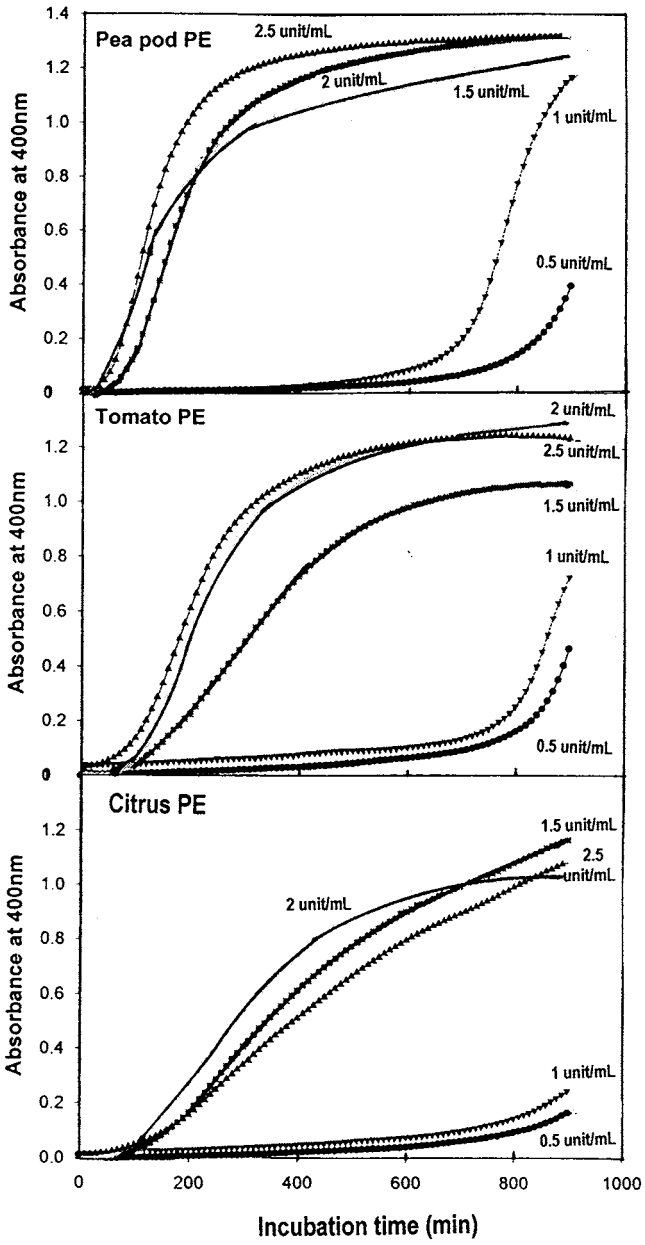


**Figure 4.** Changes in turbidity of 1.5% citrus pectin (DE = 67%)-PE mixtures (0.5, 1.0, 1.5, 2.0, and 2.5 units/mL) in 0.1 M NaCl/0.1 M phosphate buffer (pH 6.5) at room temperature for up to 15 h. The experiment was repeated 3 times with similar results.

molecules as a result of the transacylation reaction. Li et al. (*24*) and Yokotsuka and Singleton (*25*) indicated that increase in sample turbidity was relevant to the increases in molecular weight and particle size of samples. In addition, gel permeation chromatography was also helpful in proving the increase in pectin molecular weight as a result of the transacylation reaction (*2*, *3*).

Similar to that for pea pod PE, 1.5% pectin−tomato PE mixture exhibited a marked change in turbidity (about 1.5 at 800 min of incubation time) with a turbidity-initiating time (about 400 min) much longer than that (about 200 min) for 1.5% pectin−pea pod PE mixture. Citrus PE catalyzed the transacylation reaction remarkably when it was also incubated with 1.5% citrus pectin. However, its maximal absorbance at 400 nm was 1.4 at about 900 min of incubation time, apparently inferior to pea pod PE and tomato PE in catalyzing the transacylation reaction. Therefore, at the same level (2 units/mL) of enzyme activity, pea pod PE displayed the most marked turbidity increase, followed by tomato PE and citrus PE. Because 1.5% citrus pectin was determined to be most suitable for the transacylation reaction of three PEs in the present study, it was used in the following reactions with PEs.

The pH value of the pectin-PE reaction mixtures was influential on the turbidity changes. PEs from pea pod, tomato and citrus sources apparently increased the turbidity when they were incubated with pectin at pH 7.5 and 6.5 (**Figure 3**). However, like the other two PEs, pea pod PE at pH 7.5 showed a shorter turbidity-initiating time (about 100 min) than it did (about 200 min) at pH 6.5 (**Figure 2**). It revels that weak alkaline pH range is favorable for both the de-esterification (**Figure 1**) and the transacylation reactions. Gradual catalysis on transacylation reaction was observed when pea pod PE and tomato PE were at pH 5.5 (**Figure 3**). However, no change in turbidity was observed when citrus PE was at pH 5.5, although low de-esterification activity was observed under this pH value (**Figure 1**).

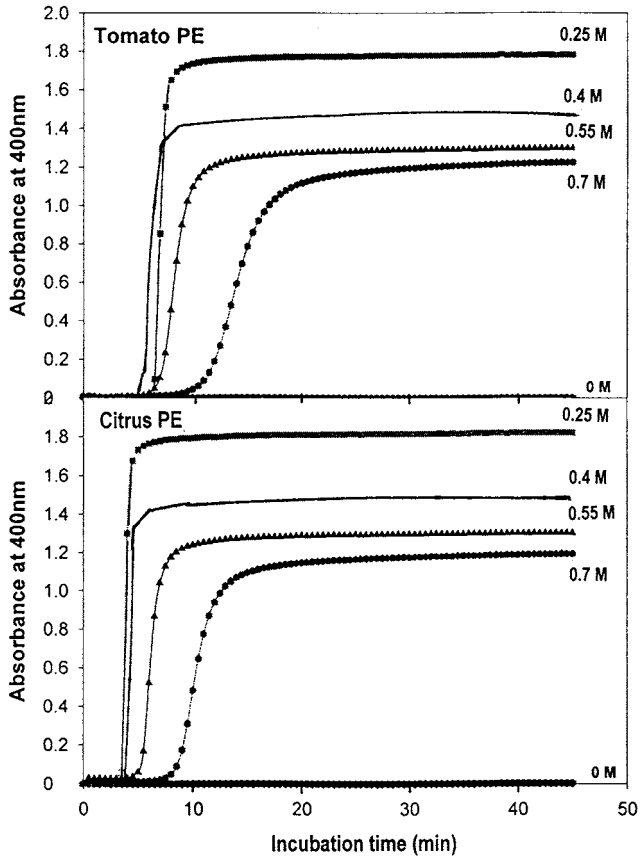


**Figure 5.** Changes in turbidity of 1.5% citrus pectin (DE = 67%)-PE mixtures (2 units/mL) in 0–0.7 M NaCl/0.1 M phosphate buffer (pH 6.5) at room temperature for up to 15 h. The experiment was repeated three times with similar results.

**Figure 4** presents the changes in PE activity in pectin−PE mixtures on the transacylation reaction. It was obvious that high PE activity (2.5 units/mL mixture) increased the turbidity promptly, revealing the fast catalysis on the reaction. However, no apparent difference in the increase of turbidity of the pectin−PE mixtures containing 2 and 2.5 units/mL were observed at the incubation time of 600 min. Among the PEs tested, pea pod PE displayed the most remarkable catalysis on transacylation reaction, followed by tomato PE and citrus PE. The causes of the difference in the extents of catalyzing such reaction were unclear, and they could be closely related to the varieties in intrinsic properties of enzymes.

For the convenience of the observation of the effect of NaCl level (0−1 M) on turbidity increase of 1.5% pectin−PE mixture, incubation time was reduced to 45 min (**Figure 5**). PE, from either tomato or citrus source, in 0.25 M NaCl exhibited the most remarkable catalysis (with a turbidity value of 1.8) on the transacylation reaction, followed by PE in 0.4, 0.55, 0.7, and 0 M NaCl. These results were similar to the observations in **Figure 1** in optimizing the de-esterification conditions for tomato and citrus PEs.

Effect of pectin DE on transacylation reaction was presented in **Figure 6**. Among the samples tested, pectin with a DE of 89% exhibited the most remarkable increase in turbidity value (about 1. 6) when incubated with pea pod PE in 0.1 M NaCl, followed by pectin with a DE of 67 and 31%. PE converts pectin to low methoxyl pectin and pectate through the formation of enzyme−substrate complexes, and therefore, pectin molecules with higher $C_6$ methoxyl content are liable to form such complexes and result in the transacylation reaction (*26*). Addition of 20 mM EDTA in the pectin−PE reaction mixture

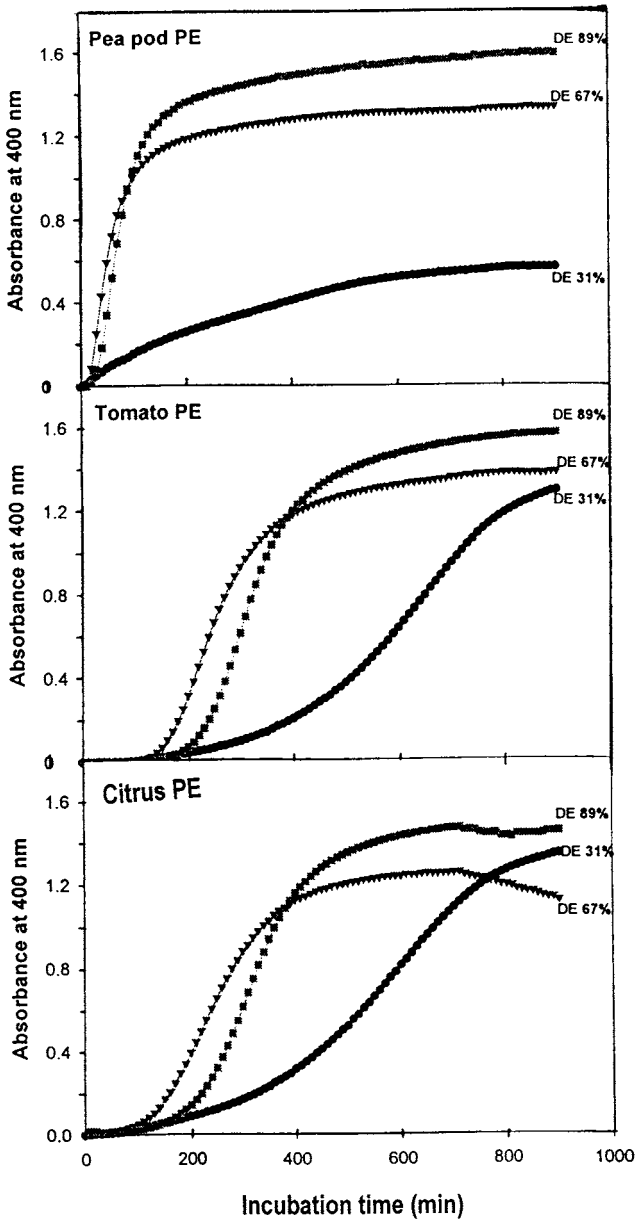


**Figure 6.** Changes in turbidity of 1.5% citrus pectin (DE = 31, 67, and 89%)–PE mixtures (2 units/mL) in 0.1 M NaCl/0.1 M phosphate buffer (pH 6.5) at room temperature for up to 15 h. The experiment was repeated three times with similar results.

was crucial to prevent the turbidity occurrence resulted from the interactions between low methoxyl pectin (DE = 31%) and divalent ions in the buffer solution during the experiment.

**Transacylation of Apple Pectin**. Apple pectin solution (1.5%) in 0.1 M NaCl at pH 6.5 was incubated with PEs from three sources at room temperature for up to 45 min to observe the turbidity change (**Figure 7**). It was obvious that the increases in turbidity (**Figure 7B**) induced by PE from pea pod, tomato, or citrus source was similar to that induced by the corresponding PE in the citrus pectin (**Figure 7A**). Therefore, apple pectin was considered to be capable of forming high molecular weight pectin compounds through the catalysis of PEs from these sources.

Thus, the optimal conditions for transacylation reactions induced by tomato PE and citrus PE were almost identical to those for de-esterification reactions. High methoxyl pectins, with DE values higher than 50%, regardless of the sources, were available for the transacylation reaction with PE.

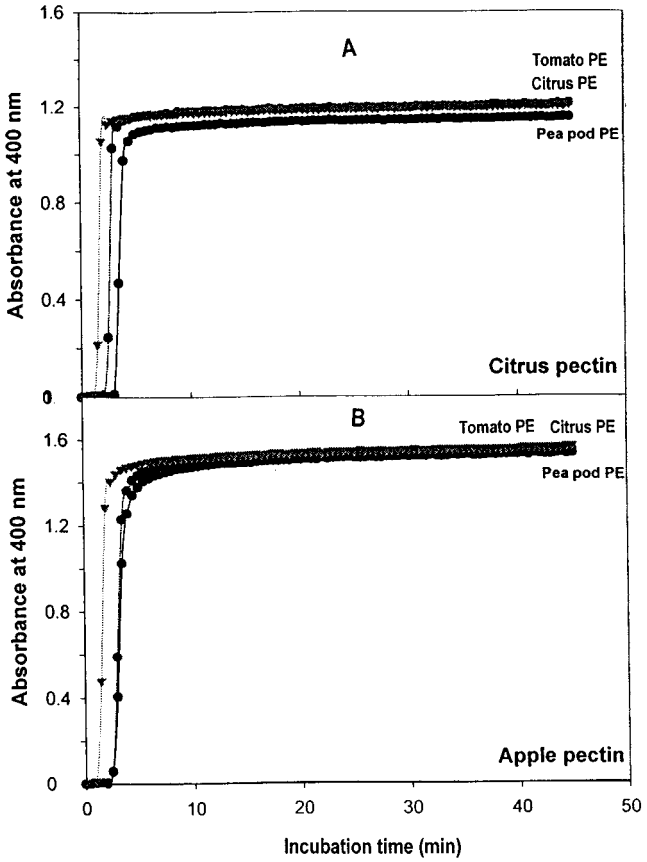


**Figure 7.** Changes in turbidity of 1.5% citrus (DE = 67%) (A) or apple pectin (DE = 73%) (B)–PE (2 units/mL) mixtures in 0.1 M NaCl/0.1 M phosphate buffer (pH 6.5) at room temperature for up to 45 min. The experiment was repeated three times with similar results.

## CONCLUSION

In addition to pea pod PE isozymes, mixtures of PE isozymes from tomato and citrus sources remarkably catalyzed the transacylation reaction to form higher molecular weight pectins through the turbidity observations on the pectin−PE reaction mixtures. The optimal temperature for the transacylation reaction of PE was undetermined. However, it was interesting to note that the optimal conditions of pH value and NaCl level for both de-esterification and transacylation reactions were almost identical. PEs from tomato and citrus fruits, the vast sources of fruits for food processing, catalyzed the transacylation reaction, and in other words, would help gelling of pectin. Further studies on the application of transacylation reaction in pectin gels will be of great interest.

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