African Journal of Food Science Vol. 5(13), pp. 710-716, 15 November, 2011
Available online at http://www.academicjournals.org/AJFS
ISSN 1996-0794 ©2011 Academic Journals
DOI: 10.5897/AJFS11.158

Full Length Research Paper

Transacylation properties of pectin methyl esterase from *Aspergillus niger*

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Accepted 24 September, 2010

Transacylation reaction of citrus pectin catalyzed by pectin methyl esterase (PME) from *Aspergillus niger* observed by laser particle size analyzer was investigated. PME was purified by a highly methoxylation of cross-linked alcohol-insoluble solids (HM-CL-AIS) column chromatography and a subsequent Sephadex G-75 column chromatography from commercial pectic enzyme (CPE) of *A. niger*. The increases of particle size (transacylation reaction) in PME-treated pectin solution are pectin content and PME activity dependent and expressed a maximum at 0.3% pectin and 0.5 U/ml PME, respectively. PME activity (de-esterification reaction) was highly stable below 50℃ but lost completely when above 70℃, while the particle size of PME-treated pectin solution decrease at temperature above 40℃. PME activity was optimum at pH 5.0, while the increase of particle size in PME-treated pectin solution reaches a maximum at pH 3.5. The different characteristics between PME from *A. niger* and the published plant PME reveal that there are at least two kinds of PME-catalyzed transacylation reaction, one for plant PME and the other for microbial PME.

Key words: Transacylation, pectin methyl esterase, *Aspergillus niger*.

INTRODUCTION

Pectin methyl esterase (Pectinesterases, PME, EC 3.1.1.11), constantly used in the wine, juice and other food industries, has been found in plants, pathogenic fungi, and bacteria as a methyl ester group hydrolytic enzyme (Gainvors et al., 1994; Giovane et al., 1996).

In plants, PMEs 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 (Tieman and Handa 1994; Wen et al., 1999; Pilling et al., 2000; Micheli 2001; Pilling et al., 2004). The PME is also required for the systemic spread of tobacco mosaic virus through the plants (Dorokhov et al., 1999; Chen and Citovsky, 2003). It removes the methyl ester groups which exist in the carboxyl moiety of galacturonic acid from pectin and produces methanol and acidic pectin which can be further digested by polygalacturonase or pectate lyase in plant cell walls (Nighojkar et al., 1995; Gaffe et al., 1997; Banjongsinsiri et al., 2004).

PME catalyzes, according to the double-displacement mechanisms, de-esterification through transferring the C_6 carboxyl groups in the pectin-PME complexes to water molecules altering the degree and pattern of methyl esterification; and the transacylation through transferring the C_6 carboxyl groups to the hydroxyl groups of another pectin molecules and resulting the formation of high molecular weight pectins with new non-methoxy ester linkages (Hou and Chang, 1996; Jiang et al., 2001a,b, 2002; Hwang et al., 2003; Lee et al., 2003; Wu et al., 2004).

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Abbreviations: PME; Pectin methyl esterase, **HM-CL-AIS;** highly methoxylation of cross-linked alcohol-insoluble solids, **CPE;** commercial pectic enzyme, **DE;** degree of esterification, **CL-AIS;** cross-linked alcohol-insoluble solid, **SDS-PAGE;** SDS-polyacrylamide gel electrophoresis.

According to Hou and Chang (1996), the PME-catalyzed transacylation reaction between pectin molecules causes an increase in the firmness of precooked vegetables. The degree of esterification (DE) and Mw are crucial factors for the functionalities of pectin (Kim et al., 2008). The molecular weight increases while transacylation take place by PME. The turbidity of pectin-PME solution increases with a formation of suspended pectin particles which is a result of the transacylation reaction catalyzed by PME (Jiang et al., 2001b; Hwang et al., 2003; Lee et al., 2003; Wu et al., 2004).

All of the PME with transacylation activity are reported from plant source, including etiolated pea seedlings (Hou and Chang, 1996; Jiang et al., 2001b), jelly fig (Jiang et al., 2001a, b, 2002), tomato and citrus (Hwang et al., 2003; Lee et al., 2003), and tendril shoots of chayote (Wu et al., 2004). No microbial PME is reported to express treansacylation activity. In addition, factors such as salt concentration, temperature, reaction time, and pH value were reported to affect the transacyltion activity of PME. The thermal stability and the difference in transacylation activity between plant PME and microbial PME has never been discussed. Hence, the commercial pectic enzyme (CPE) from *Aspergillus niger* was used as the source in this study for PME purification. Aim of the present study is to isolate PME from *A. niger* and investigate transacylation properties of the microbial PME.

MATERIALS AND METHODS

Commercial pectic enzyme (CPE) from *A. niger* was purchased from Lallemand Australia Pty. Ltd. in North Adelaide, South Austalia. Citrus pectin with a degree of esterification (DE) of 65% was purchased from Fluka Chemical Co. in Buchs, Switzerland. Sephadex G-75 gels were obtained from Amersham Pharmacia Biotech in Uppsala, Sweden. The other chemicals were of analytical grade from Sigma in St. Louis, MO, USA.

Preparation of highly methoxylation of cross-linked alcohol-insoluble solids (HM-CL-AIS)

HM-CL-AIS was performed according to the method described by Wu et al. (2007). Briefly, pea pod was homogenized for 3 min in a homogenizer in 80% (v/v) ethanol, and the homogenate was heated at 60℃ in a water bath for 2 h, and then cooled to room temperature, and filtered through a Whatman No. 1 filter paper (Maidstone, England). The obtained residues (AIS) were homogenized again with 80% ethanol, filtered, rinsed with 95% ethanol, and dried under a hood overnight. Ten grams of pea pod AIS was mixed with 150 ml of 40% dimethyl sulfoxide and 40 ml of epichlorohydrin in a 500 ml Erlenmeyer flask with gentle mixing, and then 50 ml of 5 N NaOH was added, followed by incubation at 40℃ for 2 h and filtration through a Whatman No. 1 filter paper. The obtained residues (cross-linked alcohol-insoluble solid, CL-AIS) were rinsed with distilled water, 80 and 95% ethanol, and acetone in order at room temperature. Subsequently, 20 g of pea pod CL-AIS in an Erlenmeyer flask was added slowly to 40 ml of chilled 2 N methanolic H_2SO_4 in a cold room. Then, the flask was sealed with Parafilm and aluminum foil, and the mixture was then incubated in a cold room for 6 days and filtered through a Whatman No. 1 filter paper. The obtained residues were rinsed with methanol, 80% acetone and acetone in order. After drying under a hood overnight at room temperature, the powder (HM-CL-AIS) obtained was stored in a desiccators until use.

Isolation of PME by HM-CL-AIS chromatography

PME was purified according to the method described by Wu et al. (2007) with some modification. An aliquot of commercial pectic enzyme (0.2 ml) was applied on HM-CL-AIS column (2.5 × 20.0 cm; flow rate, 30 ml/h) for separation. The column was equilibrated with either 0.01 M citric acid buffer (pH 4) and then eluted with the same buffer at 0 to 1 M Nacl gradient for PME separation. 3 ml/tube fractions were collected and assayed for the absorbance at 280 nm and PME activity. The eluted fraction containing PME activity was collected and concentrated by ultra filtration using a 10,000-molecular-weight-cutoff membrane (Amicon stirred cell model 8200; Milipore, Milford, MA, USA), and further purified by gel filtration in a Sephadex G-75 column (1.6 × 100.0 cm; flow rate, 20 ml/h) and eluted with 0.01 M citric acid buffer (pH 4) containing 0.1M NaCl. 4 ml/tube fractions were collected and assayed for the absorbanceat 280 nm and PME activity. The protein rich fraction that retained the highest PME activity was analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) and finally stained with a solution containing 0.25% Coomassie blue R-250, 50% methanol, and 10% acetic acid. The recovery (%) of PME was calculated as the ratio of total PME activity in each purification step and CPE.

Preparation of calcium-free pectin

Pectins used for transacylation reaction were treated to remove ions, especially divalent cations which may possibly exist in pectin samples according to the method described by Jiang et al. (2001a). In brief, pectin dissolved in 5 vol. (w/v) of 0.4% Na-hexametaphosphate (Sigma) was stand at room temperature for 24 h, followed by dialysis against de-ionized water for 48 h to remove the ions which may possibly exist in the pectin sample. Calcium-free pectin powder was then precipitated, filtrated and rinsed with isopropanol and acetone.

Determination of PME activity

PME activity, the de-esterification ability of PME, was determined according to the method described by Jiang et al. (2001a). PME activity was measured in a pectin solution (25℃) containing 0.1 M NaCl and 0.5% citrus pectin (DE 65%). Titration of protons released in the pectin solution was carried out with 10 mM NaOH by continuously recording of the titration using an automatic pH stat (pH-Stat Controller PHM-290, Radiometer Copenhagen, France). One activity unit of PE is defined as the amount of enzyme releasing 1 μmol of carboxyl groups per minute produced by the PE on the pectin substrate per min at 25℃. An enzyme solution previously heated in a boiling water bath for 10 min was treated as a blank.

Determination of particle size

The particle sizes of the PME-treated pectin solutions in a well-washed and dried cuvette were 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 for the analysis of PME-catalyzed transacylation.

Table 1. Purification of pectin methyl esterase from commercial pectic enzyme.

Purification step	Activity (unit)	Protein (mg)	Specific activity (unit/mg)	Fold	Recovery (%)
CPE	1687.50	41.00	41.16	1.00	100
HM-CL-AIS column	1252.63	12.55	99.84	2.43	74.23
Sephadex G-75 column	797.24	4.16	191.66	4.66	47.24

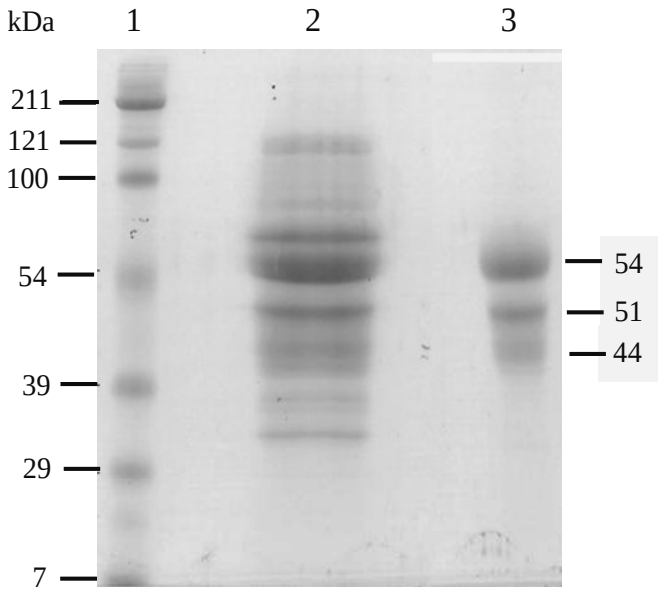

Figure 1. Protein pattern from SDS-PAGE. Lane 1, molecular weight standard; lane 2, CPE; lane 3, PME eluted from Sephadex G-75 column.

RESULTS AND DISCUSSION

Purification of PME from CPE

A PME purified from *A. niger* CPE by a HM-CL-AIS column chromatography and a subsequent Sephadex G-75 column chromatography was assayed to be with a specific activity of 191.7 units/mg. The purification results were summarized in Table 1, which were started with CPE, and resulted in 4.66 fold purification with 47.24% recovery. In addition, a major protein band with molecular mass of about 54 kDa and two minor bands (51 and 44 kDa) was observed in a SDS-PAGE (Figure 1). The molecular mass of the purified PME is higher than that of tomato, citrus, banana, and tendril shoots of chayote but similar to that of papaya (Lim and Chung, 1993; Wu et al., 2004); but lower than the molecular mass of neutral and basic PME isoforms (110 kDa each) from flax (Al-Qsous et al., 2004). The molecular mass of recombinant PME from *A. niger* strain RH5344 and *Aspergillus aculeatus* was reported as 43 kDa (Khanh et al., 1991) and 36.2 kDa (Christgau et al., 1996), respectively. It is suggested that 54 and 51 kDa bands of the isolated PME in this study are novel isoforms of 43 KDa in *A. niger*. The fraction of Sephadex G-75 column exhibiting PME activity was pooled for the following transacylation properties study.

Effects of pectin content and PME activity on the particle size

Figure 2A shows the effect of pectin content on the change of particle size of PME-treated pectin solution. The particle size of citrus pectin used as starting material was mainly distributed between 720 and 950 nm (diameter), as detected by a laser light scattering particle size analyzer. However, after reaction with PME at pH 4.5, for 4 h, the diameters of particle sizes in PME-treated pectin solution increased to 2,646~3099 nm among 0.1 to 0.5% pectin content. Since the increase of particle size in pectin-PME solution is indicated as transacylation related (Jiang et al., 2001b), larger pectin molecules are formed during incubation and the increase in pectin molecular weight is resulted from transacylation reaction between pectin molecules. During the process, the particle size of PME-treated pectin solution increased according to the amount of pectin content and expressed a maximum at 0.3% pectin.

The effect of PME activity on the change of particle size of PME-treated pectin solution was shown in Figure 2B. The particle size of PME-treated pectin solution increased according to the amount of PME and expressed a maximum at 0.5 U/mL PME. According to the PME catalytic reaction, de-esterification of pectin was determined and expressed as PME activity while transacylation was determined and expressed as changes in gel compression force, viscosity, molecular mass, turbidity, and particle size (Wu et al., 2004; Jiang et al., 2001b). Increase in viscosity and turbidity became remarkable as the level of PME from tendril shoots of chayote in pectin solution increased (Wu et al., 2004). However, the particle size of PME-treated pectin solution decreases in the presence of too much *A. niger* PME (for example, a maximum at 0.5 U/ml). It revealed the distinction of transacylation reaction catalyzed by the PME from either tendril shoots of chayote or *A. niger* is different.

Depending on the source of PME, the enzymatic hydrolysis displayed either random or blockwise distribution of deesterified galacturonic acids on pectic biopolymer (Ralet et al., 2001). Fungal PMEs from

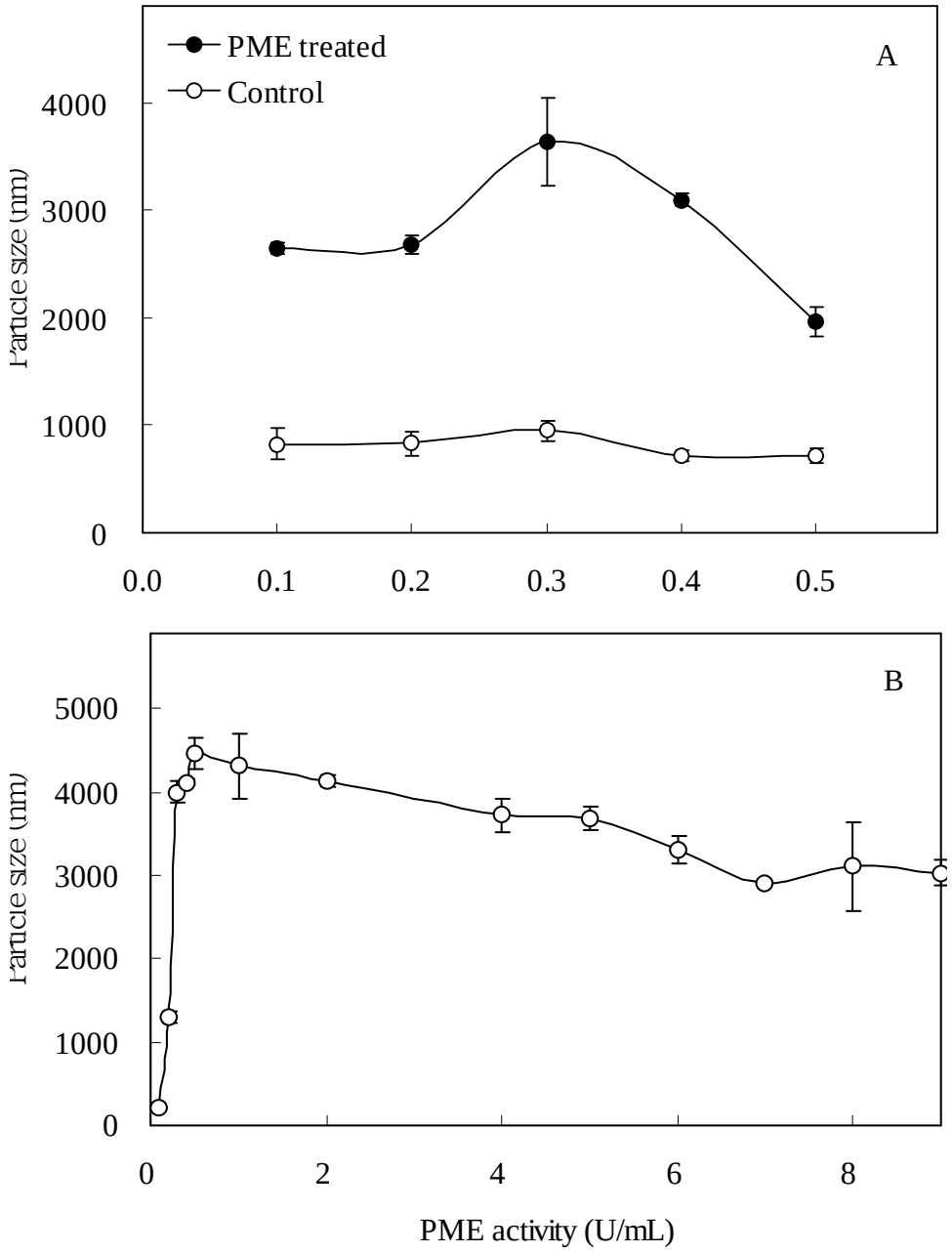

Figure 2. Effect of pectin content (A) and PME activity at 0.3% pectin (B) on the particle size of PME-treated pectin solution. The reaction was taken place at pH 4.5, 40℃ for 4 h.

Aspergillus species with acidic pH optima is believed to cleave the methoxyl groups of pectins in a random manner of de-esterified galacturonic residues, while PMEs from plants with pH optima in the neutral to alkaline region produced blockwise arrangement of free carboxyl groups (Bordenave, 1996). Therefore, the difference in PME-catalyzed transacylation reaction between PME from either tendril shoots of chayote or *A. niger* suggests that the PME-catalyzed transacylation by random attack of PME on pectin causes a decrease in particle size at high PME concentration.

Effect of temperature on the activity and stability

The optimal hydrolysis temperature of PME was observed at 40℃ (Figure 3A). The distribution of particle size increased to a maximum at 40℃ in the presence of PME,

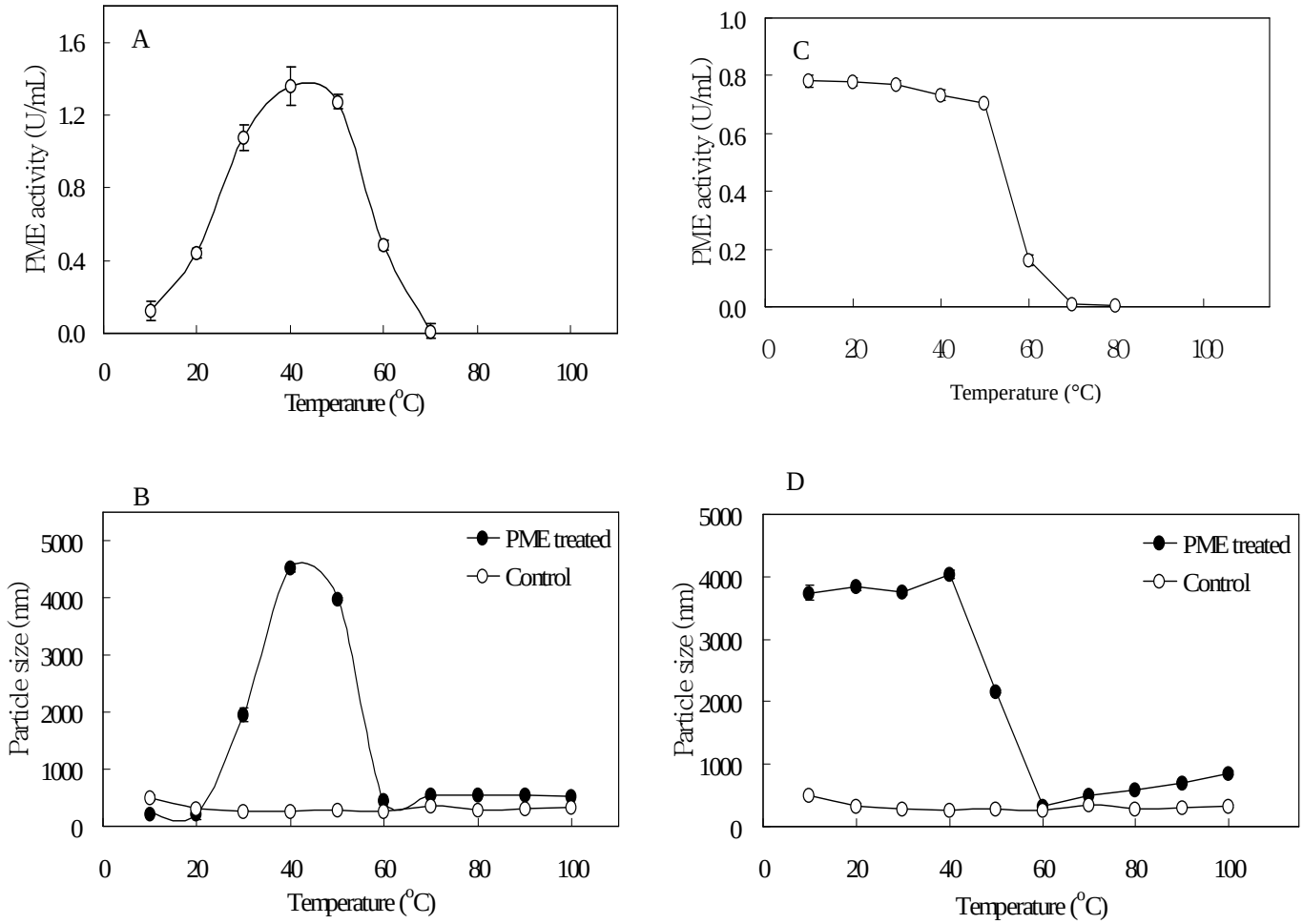

Figure 3. Effect of temperature (A, B) and thermal stability (C, D) on the changes of PME activity (A, C) and particle size (B, D) in PME-treated pectin solutions. Reaction of PME (0.3 U/mL) and pectin (0.3%) solutions for the change of particle size was taken placed at 40℃, pH 5.0 for 4 h. Control: PME was inactivated in boiling water for 10 min before incubating with pectin.

no change in the particle size of PME-treated pectin solution was observed while PME was pre-heated (Figure 3B). It is consistent with the result of PME from tendril shoots of chayote (Wu et al., 2004), that the optimal temperature for transacylation reaction and de-esterification reaction of PME were indicated to be very close or similar.

Thermal stability of PME which was pre-incubated at the expected temperature (10 to 80℃) for 30 min and then immediately cooled on ice was also studied. It is obvious that PME activity was highly stable below 50℃ and lost complete above 70℃ (Figure 3C). The particle size of PME-treated pectin solution decrease at temperature above 40℃ and down to a trough at 60℃ (Figure 3D). Pectin solution catalyzed by inactivated PME showed no change in the particle size during incubation. The result indicates that the transacylation activity of PME is more thermal sensitive than its de-esterification activity.

Effect of pH and incubation time

The pH activity profile of PME was shown in Figure 4. PME was highly active in the pH range of 3.0 to 6.0 with an optimum at pH 5.0 and this *A. niger* PME displayed about 20% of maximal de-esterification activity at pH 6.0 (Figure 4A). The optimal pH was at pH 7.5 to 8.5 for the de-esterification reaction of tomato PME and citrus PME, and almost identical to those for the transacylation reaction (Hwang et al., 2003), while PME from *A. aculeatus* had optimal activity at pH 4.5. The citrus PME had relatively broad pH activity range and at pH 5.0, this enzyme still displayed about 57% of maximal activity. The PME from *A. aculeatus* displayed a very narrow pH activity range from pH 3.5 to 5.5 and did not show any detectable activity at pH 6.0 or above (Yoo et al., 2009). It is obvious that the pH sensitivity of *A. niger* PME is similar to the results of *A. aculeatus* PME.

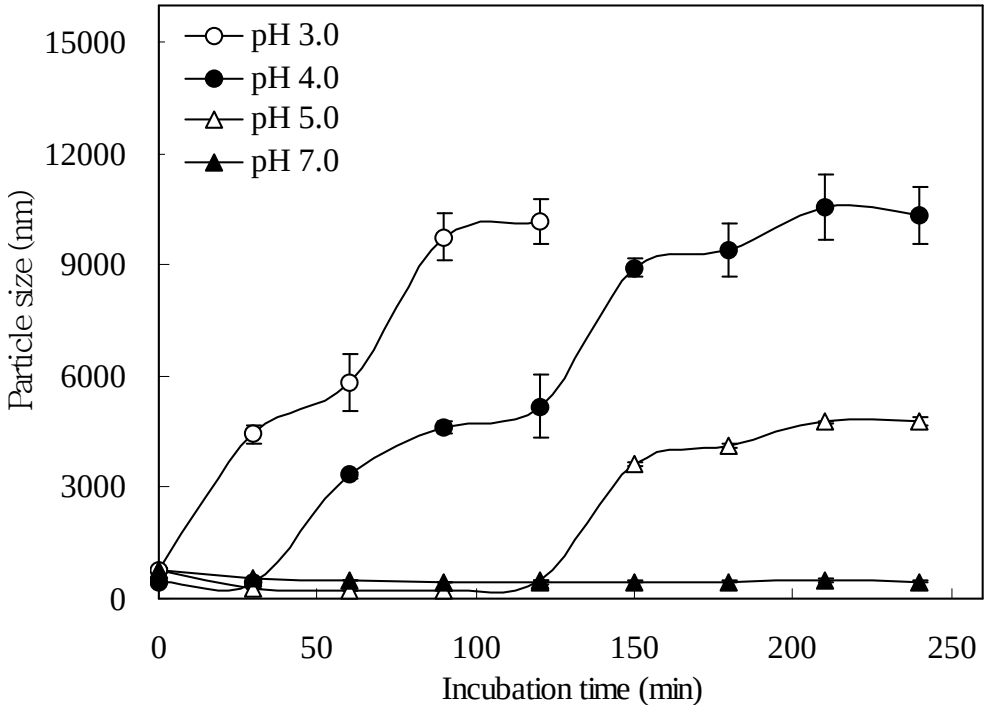

Figure 5. Effect of pH on the changes of particle size in PME-treated pectin solutions. Reaction of PME (0.3 U/ml) and pectin (0.3%) solutions was taken placed at 40℃ for 4 h

The particle size of PME-treated pectin solution was affected by pH values and reached a maximum at pH 3.5 and this enzyme still displayed about 40% of maximal transacylation activity (increase of particle size) at pH 5.0 (Figure 4B).

New ester linkage between pectin molecules (transacylation reaction) was proposed to take place starting from de-esterification of methoxyl group of pectin molecule and then transfer the galacturonic acyl group of pectin molecule from methanol to the other hydroxyl groups of intra- and/or inter-pectin molecules (Hou and Chang, 1996). Moreover, PME activity was expressed as the de-esterification activity of PME (Jiang et al., 2001a). Meanwhile, the increase of particle size in pectin-PME solution was indicated as transacylation related (Jiang et al., 2001b). In the study, the particle size of the pectin reached the maximum at pH 3.5 whereas the PME activity reached the maximum at pH 5.0 suggesting that there is equilibrium between de-esterification and transacylation reaction. Consequently, transacylation reaction reached the maximum at pH 3.5 whereas the de-esterification reached the maximum at pH 5.0.

The optimal conditions (pH value and NaCl concentration) of PMEs from tomato and jelly fig for the transacylation reaction were reported close to those for the de-esterification reaction for the corresponding enzyme (Jiang et al., 2001a; Hwang et al., 2003). However, the optimal pH of de-esterification and transacylation reaction is different. Therefore, the difference in optimal pH of transacylation reaction and de-esterification reaction between *A. niger* PME and PMEs from tomato and jelly fig indicate that there are at least two kind of PME-catalyzed transacylation mechanism.

In addition, the incubation time also affected the particle size of PME-treated pectin solution (Figure 5). The particle size of PME-treated pectin solution did not change at pH 7.0. While the particle size of PME-treated pectin solution increased and reached a plateau at 90, 210 and 210 min for pH 3.0, 4.0 and 5.0, respectively. Although the particle size decreases at higher amount of PME activity, the particle sizes of pectin molecules reach a maximum at the end of PME catalysis. The previous results indicate that the rate of PME-catalyzed transacylation varied with the pH value of enzymatic reaction.

Conclusion

PME from *A. niger* is found to catalyze the transacylation reaction and forms polymerized pectin as observed by an increase in the particle size of PME-catalyzed pectin solution. According to the results in the effects of PME activity and pH sensitivity, it is obvious that the transacylation mechanism of plant and microbial is

different, and there are at least two kind of PME-catalyzed transacylation. One for plant PMEs from including tomato and citrus and another for microbial PME from *A. niger*. Further studies on the application of *A. niger* PME-catalyzed transacylation reaction for the preparations of reduced sugar-pectin jellies and -jams will be of great interest.

ACKNOWLEDGEMENTS

Financial support from the National Science Council of the Republic of China under Grant NSC 96-2221-E-020-016 is greatly appreciated.

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