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# Commercially suitable pectin methylesterase from Valencia orange peels

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**Abstract:** A simple and effective procedure was developed to extract pectin methylesterase (PME) from Valencia orange peels. Orange peels contain 25-34 µmol of COOH $min^{-1}$ $g^{-1}$ of peel PME activity. The enzyme was ionically bound to cell walls and could not be extracted with water. This enables removal of water soluble pectic substances and oils from peels via homogenization and washing with water before enzyme extraction. Enzyme extraction can be conducted simply by addition of suitable amounts of NaCl (optimum: 10 g of NaCl 100 $g^{-1}$ of extraction mixture) to peel homogenate and stirring (optimum: 30 min at 200 rpm). The PME extracted from orange peels contains almost the same amount of heat-stable and heat-labile fraction, and the enzymes cannot be activated by mild heating. A slight activation of enzyme (almost 20%) was achieved by adding 1 mM $CaCl_2$ to enzyme extracts, but this agent was inhibitory at higher concentrations. The extracts stabilized by Na-benzoate and K-sorbate maintained more than 90% of their PME activity at 4 °C for at least 5 months. The obtained PME was successfully used to prepare low-methoxyl citrus pectin used in edible film formation in the presence of $CaCl_2$. This study shows the potential of using Valencia orange peels as a source of commercial PME.

**Key words:** Extraction, orange peel, pectin methylesterase, stability, Valencia orange

## Valencia portakal kabuklarından ticari olabilecek pektin metilesteraz eldesi

**Özet:** Bu çalışmada pektin metilesteraz (PME) enziminin Valencia portakal kabuklarından eldesi için basit ancak etkili bir yöntem geliştirilmiştir. Portakal kabukları 25 ile 34 µmol COOH $dak^{-1}$ $g^{-1}$ kabuk arasında değişen düzeyde PME aktivitesi içermektedirler. Enzim, hücre duvarına iyonik olarak bağlı bulunmakta ve su ile ekstrakte edilememektedir. Dolayısıyla bu durum enzim ekstraksiyonu öncesinde suyla homojenizasyon uygulanarak suda çözünen pektik maddelerin ve kabuk yağının ortamdan uzaklaştırılabilmesine olanak sağlamaktadır. Enzim ekstraksiyonu, kabuk homojenatına uygun miktarda NaCl eklenmesi (optimum: 10 g NaCl 100 $g^{-1}$ ekstraksiyon karışımında) ve karıştırma (optimum: 200 rpm'de 30 dak.) ile kolaylıkla gerçekleştirilebilmektedir. Kabuklardaki enzimin yaklaşık yarısı ısıya dirençli, diğer yarısı ise ısıya duyarlı fraksiyonlardan oluşmakta olup, enzim ılımlı ısıtmayla aktive olmamaktadır. Enzim ekstraktına 1 mM $CaCl_2$ ilavesi ile az da olsa bir aktivasyon (yaklaşık %20) sağlanabilmekte, ancak yüksek konsantrasyonlardaki $CaCl_2$ ilavesi enzimi inhibe edici etki göstermektedir. Na-benzoat ve K-sorbat ile stabilize edilmiş ekstraktlardaki PME, + 4 °C'de en az 5 ay süre ile aktivitesini %90'nın üzerinde korumaktadır. Elde edilen PME, $CaCl_2$ varlığında, yenilebilir film eldesinde kullanılan düşük metoksilli pektininin hazırlanmasında başarılı şekilde kullanılmıştır. Bu çalışma Valencia portakal kabuklarının ticari PME üretimi için potansiyel bir kaynak olabileceğini göstermiştir.

**Anahtar sözcükler:** Ekstraksiyon, pektin metilesteraz, portakal kabuğu, stabilite, Valencia portakalı

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## Introduction

Pectin methylesterase (PME, E.C. 3.1.1.11) catalyzes the de-esterification of pectin, and produces liberated carboxyl groups and methanol as products. PME exists in all higher plants, but it is particularly abundant in citrus fruits (Cemeroğlu et al. 2001; Johansson et al. 2002; Nielsen and Christensen 2002). In plants PME works in coordination with other pectinases to modify pectin. PME-demethylated pectin is a good substrate for polygalacturonase, and other depolymerization enzymes that degrade pectin and cause the softening of plant tissues by loosening the cell walls. This type of softening, mediated by PME, is a great problem during the processing of some fruit and vegetables; however, the most significant technological problem associated with PME action is loss of desired cloud stability in citrus juices. Cloud loss in citrus juices occurs by the interaction of negatively charged sites on demethylated pectin with positively charged divalent ions ($Ca^{+2}$ and $Mg^{+2}$) and the subsequent formation of insoluble salts of pectate, which destabilizes cloud particles (Ackerly et al. 2002). Therefore, inactivation of enzymes in citrus juices by heat treatment is necessary (Giovane et al. 2004; Guiavarc'h et al. 2005; Croak and Corredig 2006).

Although PME activity is generally undesirable during the processing of different foods, there are also many beneficial technological processes based on the reaction of this enzyme. In fact, fungal PME has been widely used in the industrial processing of fruits and vegetables to aid degradation of pectin, and facilitate extraction, liquefaction, maceration, filtration, and clarification processes (Alkorta et al. 1998; Bhat 2000; Cemeroğlu and Karadeniz 2001; Demir et al. 2001; Kashyap et al. 2001; Sarıoğlu et al. 2001; Wilin´ska et al. 2008). The use of PME in the modification of pectin to obtain superior gelling agents, in enzymatic firming of fruits and vegetables treated with $CaCl_2$, and in enzymatic peeling of fruits treated with a suitable blend of pectinases has also become very popular (Janser 1996; Pretel et al. 1997; Suutarinen et al. 2000; Kashyap et al. 2001; Pszczola 2001; Ralet et al. 2001; Schmelter et al. 2002; Suutarinen et al. 2002; Degraeve et al. 2003; Vierhuis et al. 2003; Ni et al. 2005; Guillemin et al. 2008; Laratta et al. 2008). Although plant PME is currently not used in the food industry, there are some successful experimental studies related to the application of commercial or extracted orange peel or pulp PME for the enzymatic firming of fruits and vegetables, and modification of pectin for use as a functional food ingredient (Massiot et al. 1997; Suutarinen et al. 2000; Kim et al. 2005; Lutz et al. 2009). Enzymatic firming is applied by treating fruit and vegetable products with PME and $CaCl_2$ under vacuum (Suutarinen et al. 2002). This process increases firmness by cross-linking enzyme-demethylated pectin molecules in the product with the added $Ca^{+2}$ atoms (Micheli 2001). On the other hand, enzymatic modification of pectin with PME is used to modify the degree of esterification. Modified pectins with various gelling and emulsion stabilizing properties are frequently used in the food industry as functional ingredients in different food products (Oosterveld et al. 2002; Lutz et al. 2009).

Orange peel is known as a rich source of PME (Cameron et al. 1994; Cameron et al. 1998; Cemeroğlu et al. 2001; Johansson et al. 2002; Nielsen and Christensen 2002). However, the enzyme market offers only lyophilized pure commercial preparations of orange peel PME, which are not suitable for food applications due to their very high price and ammonium sulfate content. The aim of the present study was to develop a simple and effective extraction method for PME from Valencia orange peel and to produce a commercial PME preparation in liquid form stabilized with food antimicrobials. The use of orange peel in PME production provides an additional byproduct from this agro-industrial waste, in addition to pectin production.

## Materials and methods

### Materials

Valencia oranges were purchased from a local market in İzmir, Turkey. In the laboratory orange juice was extracted with a manual extractor and the peels were frozen and kept at –18 °C until used in the experiments. Citrus pectin (galacturonic acid content 79%, methoxy content 8%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium benzoate and potassium sorbate were purchased from AppliChem (Darmstadt, Germany). All other chemicals used were reagent grade.

**PME extraction**

To extract PME enzyme, 5 different extraction procedures were tested. In the first extraction procedure (no. 1), orange peel (30 g) was homogenized with (90 mL) cold distilled water. Homogenization was conducted for 3 min in a Waring blender. After filtration with cheese-cloth, the pellet was discarded and the supernatant was centrifuged at 3000 ×g for 15 min at 4 °C and was used as the enzyme extract. In the second extraction procedure (no. 2), instead of cold distilled water, 1 M NaCl solution was used for the homogenization of orange peel. The enzyme extract was then obtained by applying cheese-cloth filtration and centrifugation, as described in the first extraction procedure. The supernatant was used as the enzyme extract. In the third extraction procedure (no. 3), orange peel (30-50 g) was homogenized with cold distilled water (150 mL) and filtered with cheese-cloth. The filtrate was then discarded and the collected pellet was once more homogenized with cold distilled water. Following the second cheese-cloth filtration, the pellet was mixed with different amounts of NaCl and the final weight of mixture was made to 100 g with distilled water (the final NaCl content in the extract varied between 0 and 12 g of salt 100 $g^{-1}$ of extract). The mixture was then stirred for different periods of time at 4 °C (0-240 min) for the extraction of the enzyme, filtered with cheese-cloth, and centrifuged at 3000 ×g for 15 min at 4 °C to obtain a clear enzyme extract. In the fourth extraction procedure (no. 4), the third procedure was used, but 2 g of PVPP was used as phenolic scavenger during extraction of the pellet by using NaCl (6 g of salt 100 $g^{-1}$ of extract). In the fifth extraction procedure (no. 5), orange peel (30 g) was first homogenized with cold acetone at −18 °C (200 mL) to remove phenolic compounds. The homogenate was then filtered with a Buncher funnel containing Whatman no. 1 filter paper under vacuum to collect the pellet (the acetone was discarded). The pellet was homogenized twice with cold distilled water (2 × 150 mL) and collected by filtration through cheese-cloth. The pellet was then extracted using NaCl (6 g of salt 100 $g^{-1}$ of extract) without using PVPP, and then was filtrated and centrifuged, as described in the fourth extraction procedure. The PME activity of all enzyme extracts was determined titrimetrically.

**Determination of PME activity**

All PME enzyme activity was measured using the titrimetric method, except the effect of $CaCl_2$ on enzyme activity, which requires the use of spectrophotometry in order to prevent changes in the reaction mixture volume during activity measurement. A slightly modified version of the method given in Yemenicioğlu (2002) was used to measure enzyme activity at 30 °C; enzyme activity was expressed as percentage of initial activity or μmol of liberated COOH $min^{-1}$ $mL^{-1}$ of extract (or per gram of peel) for the given reaction mixture volume. The reaction mixtures were formed by mixing 1 mL of enzyme extract with 20 mL of 0.5% pectin solution prepared in 0.1 N NaCl. All activity measurements were performed as 3 replicates and averages were calculated.

For spectrophotometric assays the method given in Hagerman and Austin (1986) was slightly modified. The reaction mixture was formed by mixing 2.3 mL of 0.3% pectin solution prepared in 0.1 M NaCl, 0.5 mL of 0.01% (w $v^{-1}$) bromothymol blue prepared in 0.003 M sodium phosphate buffer at pH 7.5, and 0.1 mL of crude enzyme. The decrease in absorbance at 620 nm was monitored using a Shimadzu 2450 spectrophotometer equipped with a constant temperature cell holder working at 30 °C. Enzyme activity was determined based on the slope of the initial linear portion of absorbance versus the time curve and was expressed as percentage of initial activity. All activity measurements were performed as 3 replicates and averages were calculated.

**The effect of heating the peels on PME activity**

To determine the effects of mildly heating the peels on the activity of extracted PME, small pieces of orange peel (obtained from 16 peel halves from different oranges) were put into cheese-cloth sacks and incubated between 30 and 55 °C for 30 min in a circulating water bath. At the end of the incubation period the peels were cooled in cold water. Then PME was extracted from the peels using extraction procedure no. 3 under optimum conditions (using 10 g of NaCl and a 30-min stirring period during extraction) and tested for enzyme activity titrimetrically. Activity of the enzyme extracted from peels incubated at 30 °C for 30 min was taken as

100%, whereas activity of the enzymes extracted from peels heated at higher temperatures was given as percentage of residual activity.

**The effect of $CaCl_2$ on PME activity**

To determine the effect of $CaCl_2$ on PME activity, enzyme activity was determined in the presence of 0.75-50 mM $CaCl_2$. The enzyme extract used in these experiments was obtained with extraction procedure no. 3 under optimum conditions (using 10 g of NaCl and a 30-min stirring period during extraction). Enzyme activity was determined spectrophotometrically by adding varying concentrations of 0.1 mL of $CaCl_2$ to the reaction mixture.

**Storage stability of PME**

In order to obtain an enzyme preparation PME was extracted using extraction procedure no. 3 under optimum conditions (using 10 g of NaCl and a 30-min stirring period during extraction). To clarify the preparation the extract was incubated for 1 week at 4 °C and the precipitate formed was obtained after centrifugation at 3500 ×g for 15 min (at 4 °C). To prevent microbiological spoilage, 0.1% K-sorbate and 0.1% Na benzoate were then added to the clear enzyme extract (pH 3.8). The storage stability of the enzyme was determined by monitoring enzyme activity at 4 °C. The effect of calcium ions on enzyme storage stability was also tested by adding 1 mM $CaCl_2$ to the enzyme extract. Activity of enzyme was tested titrimetrically.

**Preparation of a pectin film with extracted PME**

Pectin films were prepared as follows: (1) 36 mL of 2% pectin solution was mixed with 2 mL of PME extract containing 10.8 μmol COOH $min^{-1}$ $mL^{-1}$ enzyme extract (activity according to the titrimetric method); (2) demethylation of pectin was conducted at room temperature until 2.1 mL of 1 M NaOH was spent in the titration; (3) 10 g of demethylated PME solution was pipetted into a glass petri dish (9.8 cm in diameter) and left to dry for 1 day at room temperature; (4) cross-linking of the film was accomplished by adding 5 mL of 1 M $CaCl_2$ onto the dried pectin film and by additional drying for 5-6 h at room temperature. At the end of the drying period the film was peeled from the petri dish and wetted to determine if an insoluble Ca-pectate structure was formed by the action of PME.

**Protein content measurement**

Protein content was determined according to the Lowry procedure using bovine serum albumin as the standard (Harris 1987). All assays were performed as 3 replicates and averages were calculated.

## Results

**PME activity in orange peel samples obtained at different times**

To determine potential variation in PME activity in Valencia orange peel, activity of enzyme was monitored in samples obtained in 5 different months. As seen in Figure 1, at the beginning, orange peels had PME activity of almost 33 μmol of COOH $min^{-1}$ $g^{-1}$ of peel. A reduction occurred in PME activity in the samples obtained in June and August, but the activity of these peels was only 14% lower than that of the average of 5 different samples (29.2 μmol of COOH $min^{-1}$ $g^{-1}$ of peel).

**The effect of extraction procedures on the activity of extracted PME**

Different methods were tested to develop a simple and effective extraction procedure for orange peel PME. PME activity in the extract obtained from the filtration and centrifugation of peel homogenate prepared with cold distilled water (extraction procedure no. 1) was very low (Table 1). The

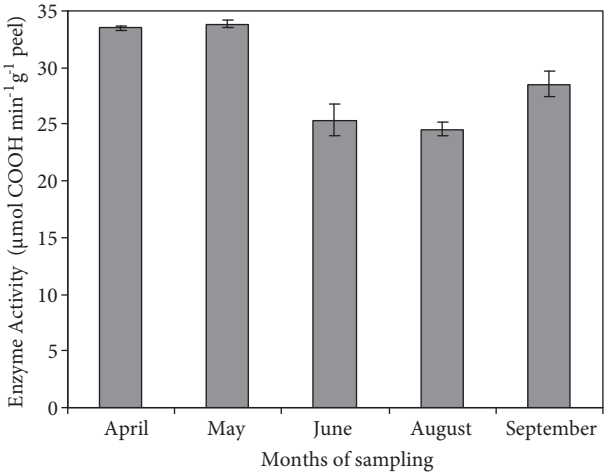


Figure 1. PME activity in Valencia orange peel samples obtained in different months (the enzyme was extracted with 1 M NaCl from the pellet obtained by homogenization of orange peels with water).

Table 1. The effect of different extraction procedures on PME activity.

| Extraction Procedure no./summary[a] | Activity obtained from peels (μmol of COOH $min^{-1}$ $g^{-1}$ of peel) |
|---|---|
| No. 1/(homogenization of peels with cold water/filtration /centrifugation/activity measurement in the supernatant) | 2.17 ± 0.22 |
| No. 2/(homogenization of peels with cold 1 M NaCl solution/ filtration/centrifugation activity measurement in the supernatant) | Gel Formation |
| No. 3/(2 × homogenization of peels with cold water/filtration/ extraction of pellet with cold NaCl solution/filtration/ centrifugation/ activity measurement in the supernatant) | 30.7 ± 0.69 |
| No. 4/(2 × homogenization of peels with cold water/filtration/ extraction of pellet with cold NaCl solution + PVPP/filtration /centrifugation /activity measurement in the supernatant) | 31.5 ± 0.35 |
| No. 5/(homogenization of peels with cold acetone/filtration/2 × homogenization of pellet with cold water/filtration/extraction of pellet with cold NaCl solution/ filtration/centrifugation/activity measurement in the supernatant) | 32.8 ± 0.81 |

[a] For extraction procedures 3, 4, and 5, 6 g of NaCl was used per 100 g of extraction mixture. All extractions were conducted in an ice water bath

homogenization of peels with 1 M NaCl solution instead of cold water (extraction procedure no. 2) also did not successfully extract the enzyme, as this caused the formation of a very thick gel in the extraction medium. With extraction procedure no. 3 the peels were homogenized twice with cold water before extraction with NaCl solution. This treatment prevented gel formation caused by homogenization with NaCl solution and facilitated extraction of PME from the washed pellet. In fact, the activity of extract obtained by salt solution from the washed peel pellet was almost 14-fold higher than that of the extract obtained directly from peels with cold water. To minimize the potential tanning effect of phenolic compounds on PME and to prevent the loss of enzyme activity due to this undesirable reaction, the addition of the phenolic scavenger PVPP during extraction of the pellet (extraction procedure no. 4) or use of acetone powder during extraction (extraction procedure no. 5) were also tested; however, these procedures did not considerably affect the activity of PME extracted from the peels.

**The effect of NaCl concentration and stirring time on the activity and specific activity of extracted PME**

Optimization of the NaCl concentration during extraction was carried out by using extraction procedure no. 3. As seen in Figure 2, when the NaCl concentration was increased from 2 to 10 g 100 $g^{-1}$ of extraction mixture, total activity of extracted PME increased accordingly. Total activity extracted was maximal when we used 10 g of NaCl 100 $g^{-1}$ of extraction mixture; however, it decreased when the NaCl concentration was further increased to 12 g 100 $g^{-1}$ of extraction mixture. Up to the 4-g NaCl concentration, increasing the salt concentration

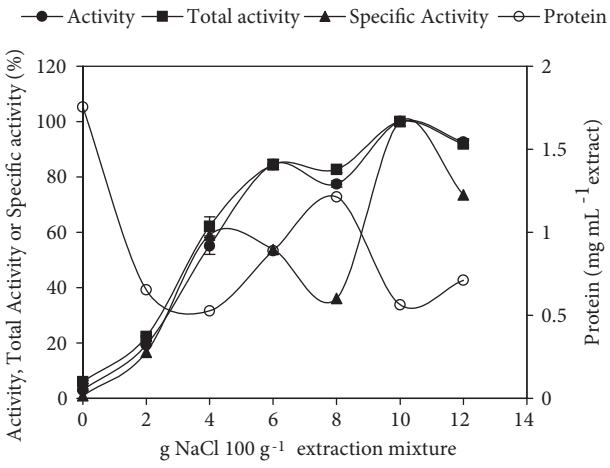


Figure 2. The effect of NaCl concentration during extraction on the activity and specific activity of PME from Valencia orange peel.

considerably increased the specific activity of the enzyme. At the 6-g NaCl concentration, the specific activity of the enzyme decreased slightly, while the 8-g NaCl concentration caused a significant reduction in specific enzyme activity. Interestingly, further increases in the NaCl concentration up to 10 g increased the specific activity of PME.

Extraction procedure no. 3 was further optimized by determining the effect of stirring time at a constant stirring rate (at 200 rpm) (Figure 3). The high initial PME activity at 0 time indicated that most of the enzyme was released from the cell walls in the presence of NaCl, without any stirring. Stirring for 15 or 30 min increased the activity of PME in the extracts by almost 50%, while further stirring caused a fluctuation, followed by a reduction in enzyme activity.

**The effect of mildly heating the peels and $CaCl_2$ on the activity of extracted PME**

To activate the PME enzyme and obtain a greater activity yield, orange peels were heated to between 30 and 55 °C for 30 min before they were used for extraction. The results given in Figure 4 indicate that orange peel PME could not be activated considerably at the studied heating conditions; however, the peak point observed at 50 °C was thought as an indication of slight activation that might occur more intensively at shorter or longer heating times. Thus, the peel heating study was once more repeated at this temperature; however, as seen in Figure 5, 15-60 min of heating at this temperature did not result in activation of the enzyme. In fact, because of the considerable amount of heat labile PME, the heating study at 50 °C yielded a biphasic inactivation curve.

In addition to mild heating, we also tested the use of $CaCl_2$ to activate the extracted enzyme. Activity measurements obtained between 0.75- and 50-mM $CaCl_2$ concentrations are given in Figure 6. As seen in this figure, slight activation of PME (almost 20%) might occur at the 1-mM $CaCl_2$ concentration. At other concentrations between 0 and 5 mM activation was not noteworthy. On the other hand, at the 10- and 50-mM concentrations, the effect of $CaCl_2$ was inhibitory.

**Storage stability of PME**

Due to very high stability, most commercial fungal PME preparations are supplied in liquid form. Thus, the stability of orange peel PME was investigated at 4 °C in liquid form stabilized using K-sorbate and Na-benzoate as preservatives (Figure 7). The enzyme extract showed good stability for 5 months, with retention of 90%-95% of its activity. No significant activation effect of $Ca^{++}$ ions was observed on PME enzyme during cold storage.

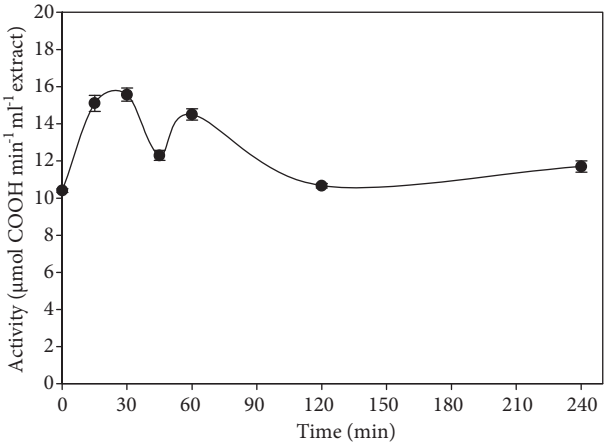


Figure 3. The effect of the extraction period on activity of PME extracted from Valencia orange peel (enzyme was extracted with extraction procedure no. 3, with 10 g of NaCl 100 $g^{-1}$ of extraction mixture).

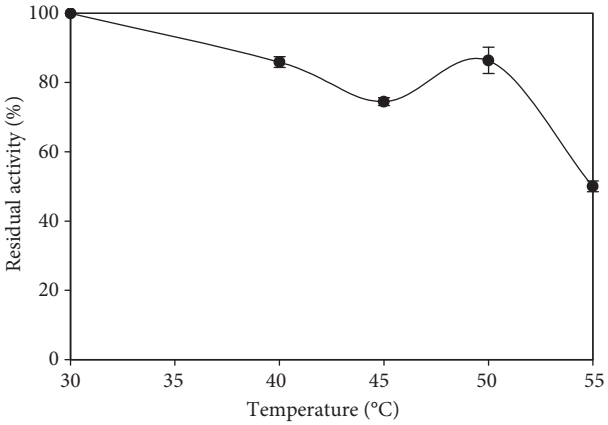


Figure 4. The effect of heating Valencia orange peel on the activity of extracted PME (heating for 30 min at different temperatures; activity of enzyme extracted from peels incubated at 30 °C for 30 min was taken as 100%).

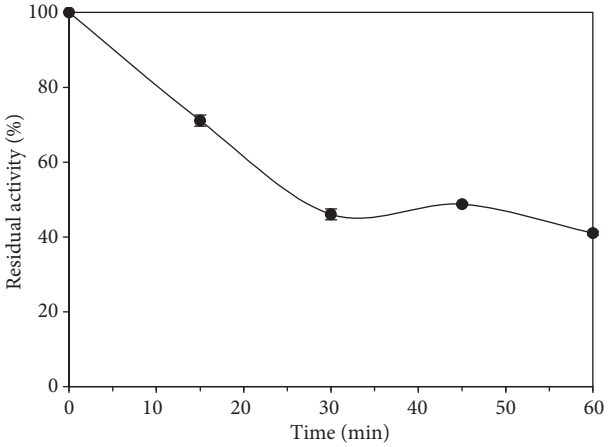


Figure 5. The effect of heating Valencia orange peel at 50 °C on the activity of extracted PME (activity of enzyme extracted from unheated peels was taken as 100%).

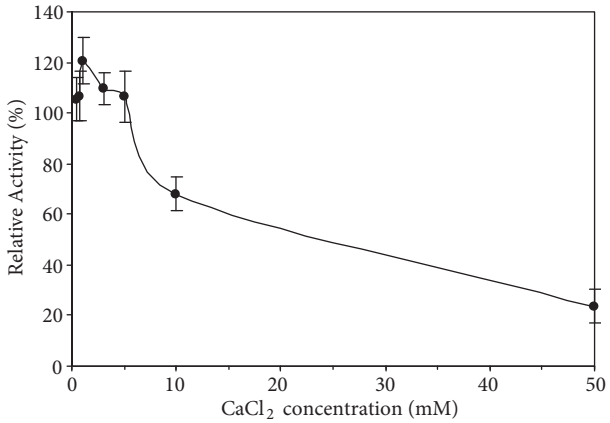


Figure 6. The effect of different $CaCl_2$ concentrations on PME activity (activity was measured spectrophotometrically).

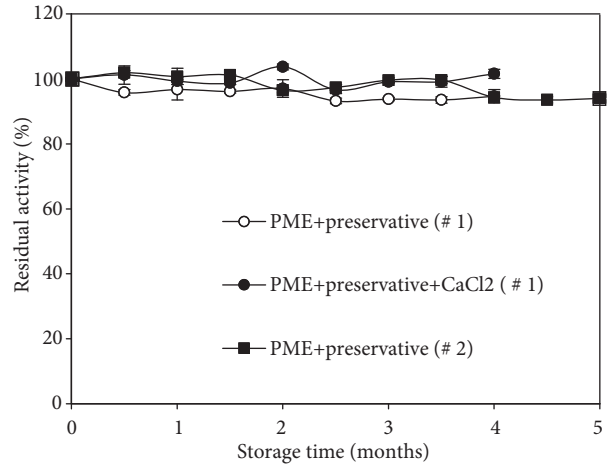


Figure 7. Stability of the prepared PME during storage at 4 °C in liquid form.

### Preparation of a pectin film with extracted PME

In the present study the action of prepared PME on commercial citrus pectin was simply confirmed by gelation and subsequent film formation testing. This test shows the ability of PME to reduce the degree of pectin methylation sufficiently to obtain a strong pectin gel in the presence of divalent ions. The effect of PME on pectin is shown in Figure 8a. The enzyme-treated pectin showed rapid gelation and formed a strong flexible film in the presence of $CaCl_2$. In contrast, the control pectin, which was not treated with PME, did not form a gel or film in the presence of $CaCl_2$ (Figure 8b). In fact, the untreated pectin was still soluble after $CaCl_2$ treatment and was easily removed from the petri dish surface.

## Discussion

### The suitability of using orange peel and the proposed extraction method

In the present study the limited change in PME activity of peel samples obtained in 5 different months clearly shows the suitability of using orange peels as a PME source (Figure 1). PME is a cell wall-bound enzyme that can only be extracted at high salt concentrations (Wicker et al. 2002; Laratta et al. 2008); therefore, it was an expected result to have no enzyme activity in cold water extracts of orange peel. Direct extraction of the ionically bound enzyme by the NaCl solution was also not possible, as this extracted both PME and soluble pectin, causing the rapid gelation of demethylated pectin in the presence of naturally occurring divalent ions. As such, the best method for extracting ionically bound enzyme was the removal of soluble pectic substances by homogenization of the peels twice with cold water before extraction with NaCl solution (extraction procedure no. 3) (Table 1). A similar extraction procedure was also used by Cameron et al. (2005) to extract PME from Valencia orange flush and mature leaves. The removal of phenolic compounds from the enzyme extract using PVPP or acetone (extraction procedures nos. 4 and 5) did not cause a significant change in the activity of PME; therefore, it is clear that the tanning effect of phenolic compounds in the obtained extracts did not cause a significant undesirable effect on PME activity.

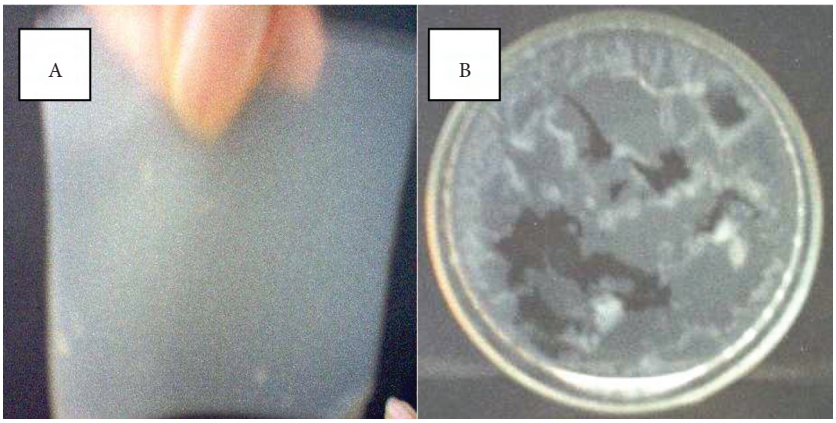


Figure 8. The effect of the prepared orange peel PME on commercial citrus pectin following $CaCl_2$ crosslinking (A: PME-treated pectin film; B: control pectin).

**Optimization of extraction conditions**

The optimization of the NaCl concentration was carried out by using extraction procedure no. 3 at different salt concentrations. As seen in Figure 2, the protein content at different salt concentrations varied greatly. Depending on these variations in protein content, specific activity calculated by dividing activity values by protein content also varied. It appears that the variation in protein content was related to changes in protein solubility due to changes in the salt concentration (Damodaran 1996). Total activity and specific activity at different salt concentrations indicate that the optimal NaCl concentration was 10 g 100 $g^{-1}$ of extraction mixture. Salt concentrations higher or lower than this were less effective, as they caused less enzyme extraction or extraction of non-enzyme proteins. Because PME is highly soluble in the presence of the optimum concentration of NaCl, most of the enzyme was extracted from the peels without any stirring (Figure 3); however, the yield increased by almost 50% by applying a stirring period of 15-30 min. A fluctuation in enzyme activity was observed at 45 and 60 min of stirring, which might have be due to limited modifications in enzyme conformation by solubilized digestive proteases. Such proteases can cause activation or inactivation, depending on the resulting modifications in the structure of the target enzymes (King and Flurkey 1987; Labuza et al. 1992; Espin et al. 1999). Longer stirring periods were not beneficial, as they reduced enzyme activity. The reduction in enzyme activity by extended stirring may have been related to more extensive changes in the enzyme structure due to digestive proteases or the release of some enzyme inhibitors from the peel tissues. Thus, to obtain maximum recovery it is essential to apply a limited extraction period at a suitable salt concentration.

**Activation of PME**

Application of mild heating might cause activation of the PME enzyme in plant tissues (Yemenicioğlu 2002; Degreave et al. 2003; Ni et al. 2005). It was thought that such activation resulted from contact of the enzyme with intracellular electrolytes after the loss of cellular membrane integrity (Yemenicioğlu 2002); however, in the present study mild heating of orange peels at different conditions did not cause activation of PME. On the other hand, heating peels at 50 °C for different time periods resulted in a heat inactivation curve, indicating the heat labile nature of the enzyme. The heat labile nature of Valencia orange peel PME was also reported by Savary et al. (2002), while Yemenicioğlu et al. (1998) and Han et al. (2000) reported the presence of both heat labile and stable forms of the enzyme in orange peel. Although the present study was not designed to investigate the heat inactivation kinetics of PME enzyme, the biphasic curve we obtained by heating peels suggests the presence of multiple isoenzyme forms of PME with varying heat stabilities. The presence of multiple isoforms of PME in Valencia orange peel was also

reported by Cameron et al. (1998) in a detailed purification study. This enabled us to calculate the percentage of heat stable and heat labile fractions of the enzyme at the studied temperature, as described by Yamamoto et al. (1962). In the procedure described by Yamamoto et al. (1962) the percentage of residual activity vs. time data was plotted on a semi-logarithmic scale to obtain the biphasic inactivation curve. Then the intercept of the second part of the biphasic curve, having a lower slope than the first part and representing the heat stable portion, was determined on the y-axis. This intercept corresponds to the percentage of the heat stable portion, while the heat labile portion was calculated by subtracting this value from total activity (100%). The percentage of the heat labile and heat stable enzyme fractions of PME calculated with this method at 50 °C are quite similar and almost 50% for each fraction.

It is well known that cations such as $Ca^{++}$ ions may also activate the PME enzyme. It was reported that the activation of PME by cations is related to the competitive displacement of PME bound to blocks of carboxylic groups on pectin. As a result of this competition, PME becomes free for further catalysis of pectin (Alonso et al. 1996; Leiting and Wicker 1997; Sun and Wicker 1999); however, our activity measurements indicated only a slight activation of the enzyme (almost 20%) at the 1-mM $CaCl_2$ concentration. At other concentrations activation was negligible or its effect was inhibitory. Similar to the proposed activation theory discussed above, the inactivation by high concentration of cations is also explained by the change in competition between the enzyme and cations for free carboxylic acid groups (Nari et al. 1991). Thus, during storage or commercial applications of orange peel PME the concentrations of $Ca^{++}$ should be chosen carefully.

**Storage stability and film test**

The high stability of the obtained PME in liquid form confirms the possibility that commercial orange peel PME in this form can be used without evaporation to obtain a dry powder (Figure 7). This is an advantage of using orange peel PME as a commercial preparation, as it reduces the cost of production. The obtained enzyme also successfully acted on citrus pectin and caused its demethylation and gelling in the presence of $CaCl_2$ (Figure 8). It is essential to show the action of the obtained enzyme on citrus pectin, which is the primary source of pectin for industrial food applications.

In conclusion, Valencia orange peel contains PME activity, which showed limited variation in different samples. The enzyme can be extracted simply by salt solutions, following the removal of soluble pectin from peels via homogenization with water. The very high stability of PME in extracts stabilized with food preservatives indicates the potential of orange peels as source of this enzyme. The use of orange peel in PME production provides an additional byproduct from this agro-industrial waste, in addition to pectin production. The most potential applications of commercial orange peel PME in the food industry are enzymatic pectin modification to obtain functional food ingredients or enzymatic firming processes applied to increase the firmness of fruits to be frozen or pasteurized. Further research is needed to show the beneficial effects of the obtained orange peel PME in different food processes.

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