Partial Purification and Characterization of Pectin Methylesterase from Acerola (*Malpighia glabra* L.)

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The enzyme pectin methylesterase (PME) is present in acerola fruit and was partially purified by gel filtration on Sephadex G-100. The results of gel filtration showed different PME isoforms. The total PME (precipitated by 70% salt saturation) and one of these isoforms (fraction from Sephadex G-100 elution) that showed a molecular mass of 15.5 ± 1.0 kDa were studied. The optimum pH values of both forms were 9.0. The total and the partially purified PME showed that PME specific activity increases with temperature. The total acerola PME retained 13.5% of its specific activity after 90 min of incubation at 98 °C. The partially purified acerola (PME isoform) showed 125.5% of its specific activity after 90 min of incubation at 98 °C. The K_m values of the total PME and the partially purified PME isoform were 0.081 and 0.12 mg/mL, respectively. The V_{max} values of the total PME and the partially purified PME were 2.92 and 6.21 μmol/min/mL/mg of protein, respectively.

INTRODUCTION

Acerola fruit is rich in vitamin C as well as carotene, thiamin, riboflavin, niacin, proteins, and mineral salts, mainly iron, calcium, and phosphorus. Acerola has been used as a remedy against flus and colds, pulmonary disturbance, liver ailments, and irregularities with the gall bladder (*1*). Used in high doses it has beneficial effects on viral hepatitis and varicella as well as poliomyelitis (*2*). Acerola is rich in antioxidant activity, which may be due in part to its high vitamin C content (*1*). Its main use is in the production of juices, concentrated pulps, and vitamin C. Acerola has also been shown to exhibit active antifungal properties (*2*).

In acerola are also present pectic substances. Pectic substances (pectins) in plant tissues and enzymes that degrade them are of major importance to the food industry because of their effect on the texture of foods, such as apples, peaches, and tomatoes, and in the preparation of wines and fruit juices (*3*).

The enzyme pectinmethylesterase (PME; EC 3.1.1.11) catalyzes the hydrolysis of the methyl ester groups from pectin and has been found in plants as well as in pathogenic fungi and bacteria (*4*). The decrease in the degree of methylation of pectin may trigger different processes related to texture and firmness (*5*). These processes may comprise cross-linking with Ca^{2+},

increase of hydration at the demethylated sites, enhancement of shielding and repulsion forces by the electric charges within the biopolymer matrix of the cell wall, decrease of the susceptibility of pectins to heat-induced β-degradation, and increase of susceptibility to polygalacturonase (PG)-induced depolymerization (*5*).

PME is of significance to the citrus industry because it has been established as the causative agent for juice clarification and gelation of frozen concentrates (*6*). In fruits, PME normally exists as two or more isoforms, each comprising a single polypeptide chain with molecular masses ranging from 10 to 60 kDa (*7, 8*), with isoeletric points between 7 and 11. Normally, this enzyme is a glycoprotein formed by a single low molecular mass polypetide (*9*). Generally, they differ in optimum pH, thermal stability, and other physicochemical characteristics (*6, 10−14*). The control of pectinesterase activity has been a common subject of study because of the implications in the modification of the texture of fruits and vegetables (*8, 9, 12, 14*) and as a destabilizing agent of pectin materials in fruit juices and concentrates.

In this work, we describe the partial purification and some physicochemical properties of total and partially purified acerola PME.

MATERIALS AND METHODS

Preparation of Pulp. Fruit samples in the mature stage of development were collected from several trees. They were collected randomly from different points of the same tree to guarantee the representability

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of the sample, because of the influence of the seasonal time in fruit development. The acerola pulp was obtained by passing the fruits through a depulper with a propeller blade. The acerola sample that was studied corresponded to 30 or more fruits, all mixed, in the ripe mature stage of fruit development (red color) according Assis et al. (*15*).

Extraction of the PME. The enzyme was extracted, at 4 °C, by using a 50 mM borate−acetate buffer, pH 8.3, containing 0.60 mol/L NaCl. The ratio of acerola material to extractant was 1:3 (g/mL). The homogenate was squeezed through two layers of gauze, and the extract was centrifuged at 10000*g* for 10 min to remove the solid particles (*16*). The precipitate was discarded. The supernatant was brought to 70% saturation by addition of solid ammonium sulfate and centrifuged at 10000*g* for 10 min after standing for 1 h. The precipitate was resuspended in borate−acetate buffer in the ratio of 1:3 (w/v). This sample was called total PME.

Purification of Acerola PME. Two milliliters of the extract (∼3 mg of protein/mL) was eluted onto a Sephadex G-100 column (48 × 1.2 cm), previously equilibrated with borate−acetate buffer, pH 8.3, plus 150 mmol/L NaCl, which was also used to elute the column. Fractions of 1.9 mL were collected and assayed for PME activity. One of these fractions, having a higher value of specific activity, was called partially purified PME.

Determination of PME Activity. PME activity was measured titrimetrically by determining free carboxyl groups formed as a result of enzyme action in pectin. The reaction mixture was composed of 15 mL of the 0.25% citrus pectin (from Sigma Chemical Co.) solution, 0.15 mol/L NaCl, and 0.5 mL of PME enzyme (total or partially purified form), and the final volume was adjusted to 30 mL with distilled water. The volume of 0.1 mol/L NaOH required to maintain the reaction mixture at pH 8.3 (27 ± 2 °C) was measured according to the method described by Kertsz (*17*). One unit of PME was defined as the amount of enzyme that released 1 μmol of carboxyl groups/min. PME activity was calculated by using the following formula (*18*):

$$\text{PME units/mL} = \frac{(\text{mL of NaOH})(\text{molarity of NaOH})(1000)}{(\text{time})(\text{mL of sample})}$$

Protein Determination. Protein concentration was determined according to the method of Hartree (*19*), using bovine serum albumin (Sigma Chemical Co.) as a standard.

PME Specific Activity Determination. The specific activity per gram of pulp was calculated by using the following formula:

specific activity (units g^{-1}/g of pulp) =

units/(g of total protein × g of pulp)

pH Optimum Determination. The pH dependence of PME activity was assayed at pH 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. Blanks without PME sample were made for each determination, and the amount of acid produced due to the spontaneous pectin demethylation was subtracted.

Temperature Optimum Determination. Temperature optimum determination on PME activity was tested under standard assay conditions at various temperatures. The temperatures (50, 60, 70, 80, and 90 °C) were controlled by means of a circulating water bath. Blanks without PME sample were made for each determination, and the amount of acid produced due to the spontaneous pectin demethylation was subtracted.

Heat Stability of Acerola PME. The samples of total or partially purified PME in test tubes (selected to be equal in weight and size) were incubated in a water or oil bath at different temperatures and for various times. After the heating process, the tubes were cooled in melting ice and the residual activity was measured. The effect of temperature on the residual PME activity was determined at 98 °C for various incubation times (10, 20, 30, 60, and 90 min).

Effect of Cations. The effect of Na$^+$, K$^+$, Mg^{2+}, and Ca^{2+} ions on PME activity was studied. The concentrations used were as follows: NaCl, 50, 100, 150, 200, 300, 400, and 500 mmol/L; KCl, 50, 100, 150, 200, and 300 mmol/L; MgSO$_4$ 12.5, 25, 37.5, 50, 75, and 1000 mmol/L; Na$_2$SO$_4$, 16.7, 33.4, 50, 66.7, 100, and 200 mmol/L; K$_2$SO$_4$,

Table 1. Extraction and Purification of PME

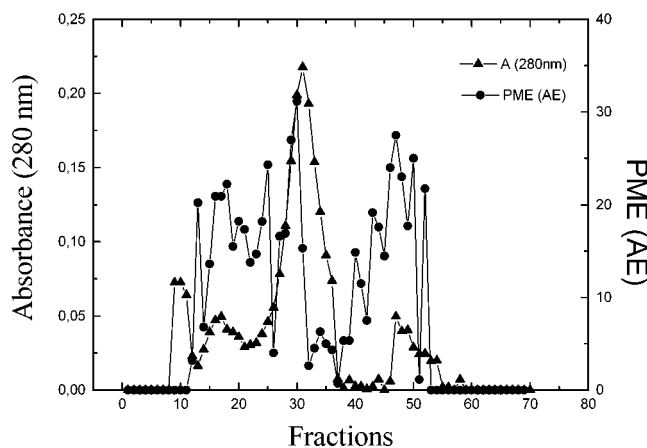| purifn step	activity units (AU)	protein (mg/mL)	specific activity (AU/mg of protein)	purifn factor
crude extract	5.32	3.78	1.41	1
ammonium sulfate precipitation (total PME)	68.16	9.26	7.36	5.2
Sephadex G-100 (fraction 31 PME)	24.93	0.64	38.95	27.6

Figure 1. Purification of PME from acerola on Sephadex G-100 column: (●) PME activity; (▲) protein content.

16.7, 33.4, 50, 66.7, 100, and 200 mmol/L; MgCl$_2$, 16.7, 33.4, 50, 66.7, 100, and 200 mmol/L; CaCl$_2$, 16.7, 33.4, 50, 66.7, 100, and 200 mmol/L.

Kinetic Parameters of PME. The effect of substrate concentration was studied by determination of initial rates (V_0) of PME activity in the presence of various substrate concentrations (1, 0.75, 0.5, 0.25, 0.125, 0.075, and 0.05 mg/mL). The K_m values and the maximum rates (V_{max}) were determined using the Lineweaver−Burk double-reciprocal plot in which the reciprocals of the initial rates of the PME activity were plotted against the reciprocals of the pectin concentrations used.

Molecular Mass Determination. The molecular mass determination of acerola PME was estimated by gel filtration on Sephadex G-100, according to the method of Whitaker (*20*). The column was calibrated for molecular mass by determining the elution volumes of standard proteins [bovine serum albumin (66000 Da), ovalbumin (45000 Da), and cytochrome *c* (12400 Da] and tannic acid (polymer, 1852 Da). The proteins and the tannic acid were dissolved in borate−acetate buffer and applied to the column, and 2.0 mL fractions were collected. The void volume was determined with a Blue Dextran 2000 solution.

Statistical Analyses. Average values of triplicates (which differed by <5%) were calculated. The data obtained from the studies were analyzed using linear or quadratic regression.

RESULTS

Purification of PME from Acerola. PME was extracted from acerola as described above. Ammonium sulfate precipitation of the crude enzyme resulted in a 5.2-fold increase in specific activity. Subsequently, the PME was eluted on a Sephadex G-100 column equilibrated with borate−acetate buffer, pH 8.3. The results of this purification step are summarized in **Table 1**. Elution results in 11 peaks of PME activity are shown in **Figure 1**. We chose the PME isoform (fraction 31) that shows the higher specific activity for further characterization and to verify the different properties for further immobilization assay. This step showed a 27.62-fold purification (**Table 1**).
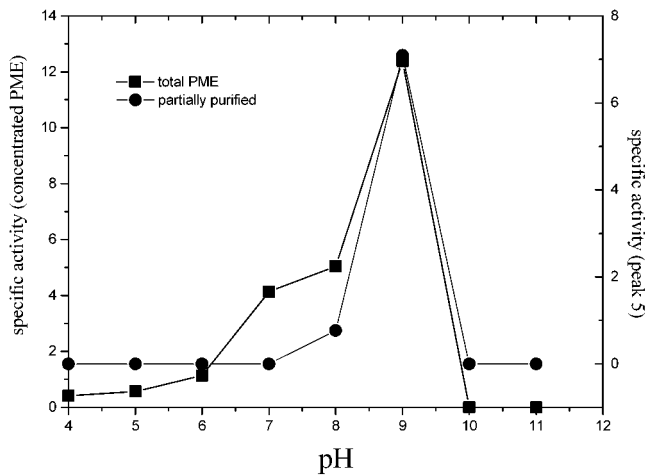
Figure 2. Optimum pH determination in the activity of PME. PME is assayed in 0.15 M NaCl, pH range 4–11, 45 °C, 0.125% citrus pectin.
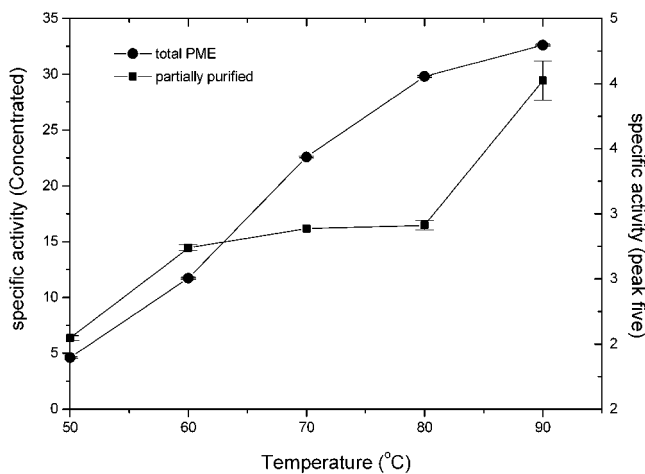

Figure 3. Temperature optimum of total or partially purified acerola PME. All reactions were carried out at pH 8.3, with 0.125% citrus pectin and 0.15 M NaCl.

pH Optimum Determination. The values of specific activity of total or partially purified acerola PME showed a single optimum value of pH 9.0 (**Figure 2**).

Temperature Optimum Determination. The effect of temperature on the specific activity of acerola PME is shown in **Figure 3**. The total and partially purified acerola PME showed that PME specific activity increases with temperature studied.

Heat Stability of PME. The effect of temperature on the residual specific activity of total or partially purified acerola PME is shown in **Figure 4**. For both, results show high stability at 98 °C. The total acerola PME retained 13.49% of its specific activity after 90 min of incubation at 98 °C. The partially purified acerola (**Figure 1**) PME showed 125.54% of its specific activity after 90 min of incubation at 98 °C.

Effect of Cations. The effects of Na^+, K^+, Mg^+, and Ca^{2+} ions on PME activity are shown in **Figures 5−7**. The specific activity was higher in the presence of 0.15 mol/L NaCl or 0.0667 Na_2SO_4 mol/L. In the presence of K_2SO_4 the specific activity was higher in the concentration range of 0.05−0.10 mol/L. In the presence of $MgCl_2$ the specific activity was high in 0.05 mol/L solution. The presence of $MgSO_4$ inhibited the enzymatic activity at all concentrations studied. In the presence of $CaCl_2$ the specific activity was high at 0.034 mol/L.

Kinetic Parameters of PME. The K_m (Michaelis constants) values of the acerola PME were determined after gel filtration
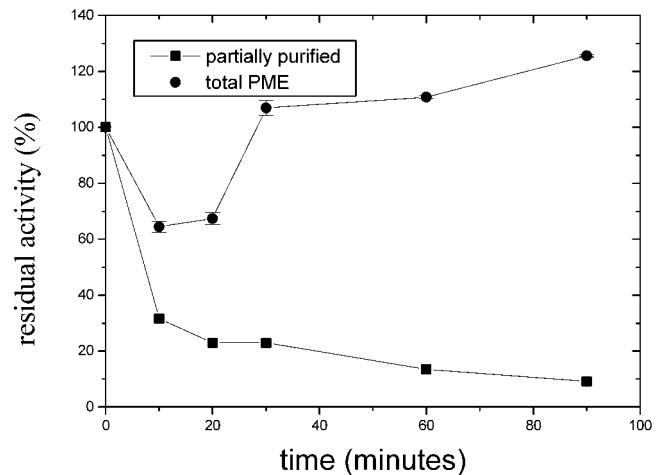

Figure 4. Heat stability at 98 °C of total or partially purified PME. All reactions were carried out at pH 8.3, with 0.125% citric pectin and 0.15 M NaCl and at a temperature of 45 °C (after storage at 98 °C for different times).
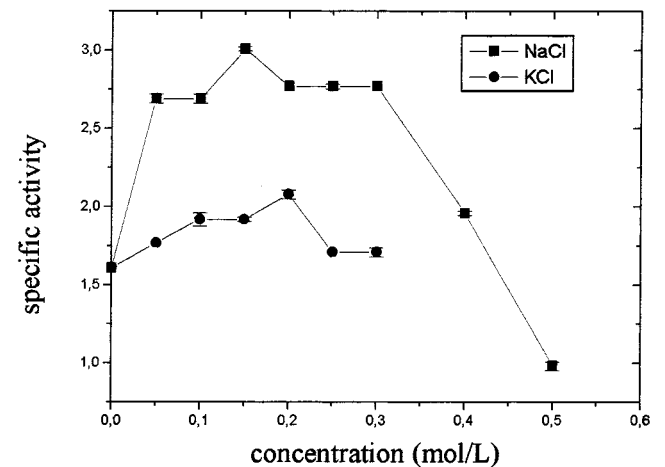

Figure 5. Effect of NaCl and KCl on acerola PME activity. All reactions were carried out at pH 8.3 and a temperature of 45 °C, with 0.125% citrus pectin.
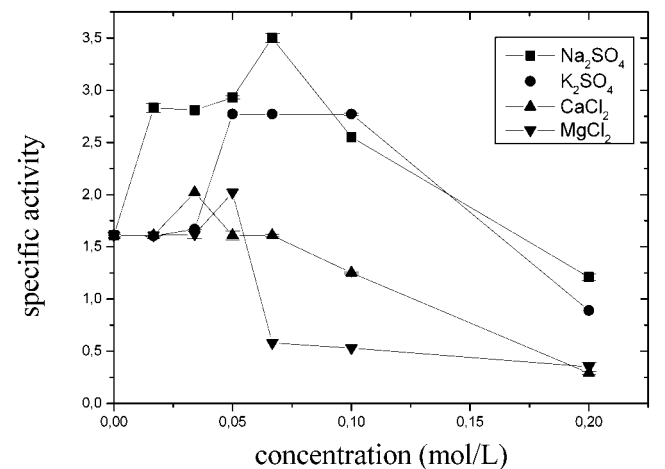

Figure 6. Effect of Na_2SO_4, K_2SO_4, $MgCl_2$, and $CaCl_2$ on acerola PME activity. All reactions were carried out with 0.125% citrus pectin at pH 8.3 and a temperature of 45 °C.

on Sephadex G-100. These values were calculated from Lineweaver−Burk double-reciprocal (1/V versus 1/[S]) plots (**Figure 8**). The K_m of total PME was 0.081 mg/mL, and the K_m of partially purified PME was 0.12 mg/mL. The V_{max} values of
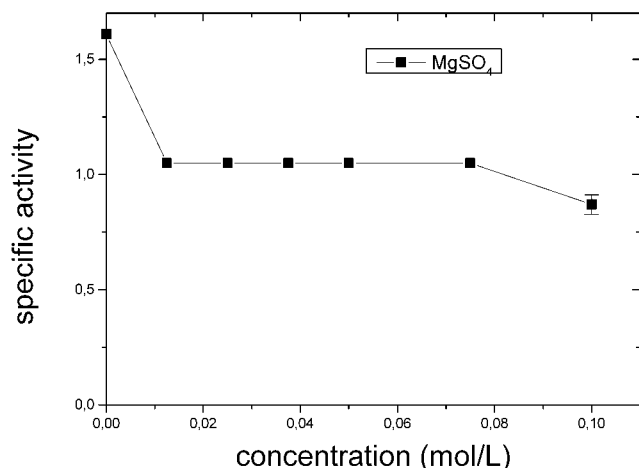
Figure 7. Effect of $MgSO_4$ on acerola PME activity. All reactions were carried out with 0.125% citrus pectin at pH 8.3 and a temperature of 45 °C.
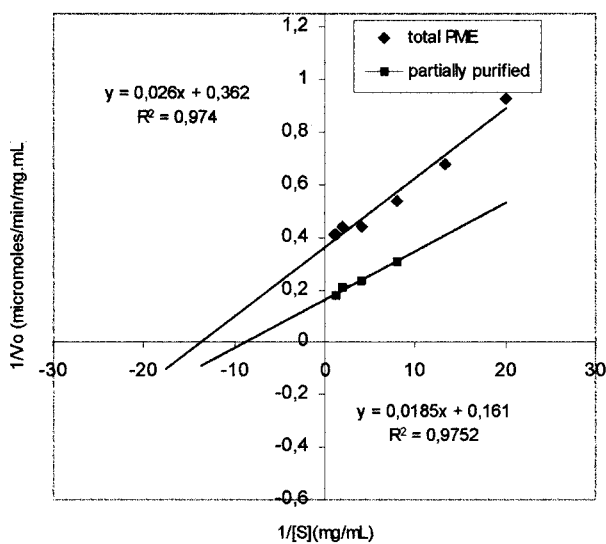

Figure 8. Lineweaver–Burk plots of acerola total PME or partially purified activity as a function of substrate concentration. Reaction mixture consisted of various concentrations of citrus pectin (1, 0.75, 0.5, 0.25, 0.125, 0.075, and 0.05 mg/mL) with 0.15 M NaCl and 0.5 mL of enzyme. The pH of the reaction was 8.3, and the temperature was 45 °C.

total PME and of the partially purified PME were 2.92 and 6.21 μmol/min/mL/mg of protein, respectively.

Molecular Mass Determination. The molecular mass of the partially purified acerola PME was determined by gel filtration on Sephadex G-100 as 15.5 ± 1.0 kDa.

DISCUSSION

The occurrence of multiple forms of PME from acerola was expected because isoforms of PME from fruits have been described by Versteeg et al. (*10*), Seymor et al. (*6, 13*), MacDonald et al. (*21*), and Cameron et al. (*22, 23*). A total of 12 isoforms have been suggested to occur in citrus (*24*). The literature showed that the higher thermally stable form of PME was found in red grapefruit and retained 45.2% of its relative stability after 60 s of incubation in a 95 °C water bath. Our results showed that (i) the total PME retained 13.49% of its specific stability after 90 min of incubation at 98 °C and (ii) the partially purified acerola PME activity increased 25.54% of its specific activity after 90 min of incubation in a 98 °C

bath. Therefore, the acerola PME (total or partially purified) is more thermal stable than the other PMEs found in fruits.

The total PME of acerola appeared as a glycoprotein by the polyacrylamide gel electrophoresis method (results unpublished). Glycosylation has been suggested as an important factor for maintaining enzyme stability (*25–27*).

The specific activity of acerola PME was higher in the presence of 0.15 mol/L NaCl or 0.0667 mol/L Na_2SO_4; we chose the concentration of 0.15 mol/L NaCl for our studies. The activation of PME by metallic ions appears to be mainly due to interaction of the ions with the substrate rather than with the enzyme (*28, 29*). A number of authors have proposed that the cleavage of the carboxylate groups adjacent to the ester bond is required to allow a correct interaction between the enzyme and the pectin (*28, 30–32*). On the other hand, because of the possibility of interactions occurring between these groups (carboxylate) with metal ions, high concentrations of the metallic ions can lead to inhibition of PME activity. Nari et al. (*27*) suggested in their studies that ionic interactions between carboxyl groups and the metal ions (high concentration) would be responsible for the inhibition of PME enzyme. Then, the metal ions are necessary for PME activity in determined concentrations, above which the ions could interfere with affinity between enzyme and substrate.

The K_m constants of total or partially purified acerola PME were of the same order of magnitude as the values found from orange (0.083, 0.0046, and 0.041 mg/mL) (*10*) and flax callus (0.147 mg/mL) (*33*). However, it is difficult to compare the K_m values found in this study with other K_m values cited in the literature because the K_m values are dependent on temperature, salt concentration, pectin source, and pH of the reaction medium (*33–35*).

Total or partially purified PME showed a single optimum value at pH 9.0. The pH optimum for plant PME isoforms is generally in a range from 7 to 9 (*30*).

In summary, acerola PME contains several isoforms. Our studies with the total form and the partially purified form showed high thermal stability. Electrophoretic analyses of the total PME suggested its being a glycoprotein molecule (results unpublished), which could explain its high stability.

LITERATURE CITED

(1) Assis, S. A.; Lima, D. C.; Faria Oliveira, O. M. M. Acerola's pectin methylesterase: studies of heat inactivation. *Food Chem.* **2000**, *71*, 465–467.

(2) Caceres, A. Plants used in Guatemala for the treatment of dermatophytic infections 2. Evaluation of antifungal activity of seven American plants. *J. Ehtnopharmacol.* **1993**, *40*, 3–9.

(3) Whitaker, J. R. Pectic substances, pectic enzymes and haze formation in fruit juices. *Enzyme Microb. Technol.* **1984**, *6*, 341–349.

(4) Giovane, A.; Quagliolo, L.; Servillo, L.; Balestrieri, C.; Laratta, B.; Loiudice, R.; Castaldo, D. Purification and characterization of three isozymes of pectinmethylesterase from tomato fruit. *J. Food Biochem.* **1994**, *17*, 339–349.

(5) Tijskens, L. M. M.; Rodis, P. S.; Hertog, M. L. A. T. M.; Proxenia, N.; Dijk, C. Van. Activity of pectin methyl esterase during blanching of peaches. *J. Food Eng.* **1999**, *39*, 167–177.

(6) Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Marshall, M. R. Purification and properties of pectinesterase of marsh white grapefruit pulp. *J. Agric. Food Chem* **1991**, *39*, 1080–1085.

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(7) Markovic, O.; Jornvall, H. Pectinesterase. The primary structure of tomato enzyme. *Eur. J. Biochem.* **1986**, *158*, 455−461.

(8) Giovane, A.; Quaglivolo, L.; Castaldo, D.; Servillo, L.; Balestrieri, C. Pectinmethylesterase from *Actinidia chinensis* fruits. *Phytochemistry* **1990**, *29*, 2821−2823.

(9) Alonso, J.; Rodriguez, T.; Canet, W. Purification and characterization of four pectinesterases from sweet cherry (*Prunus avium* L.). *J. Agric. Food Chem.* **1996**, *44*, 3416−3422.

(10) Versteeg, C.; Rombouts, K. M.; Spaansen, C. H.; Pilnik, W. Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. *J. Food Sci.* **1980**, *45*, 969−971, 998.

(11) Castaldo, D.; Quaglivolo, L.; Servillo, L.; Balestrieri, C.; Giovane, A. Isolation and characterization of pectin methylesterase from apple fruit. *J. Food Sci.* **1989**, *54*, 653−673.

(12) Lin, T. P.; Liu, C. C.; Chen, S. W.; Wang, W. Y. Purification and characterization of pectinmethylesterase from *Ficus awkeotsang* makino achenes. *Plant Physiol.* **1991**, *91*, 1445−1453.

(13) Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Wei, C.; Marshall, M. R. Stability of pectinesterases of Marsh white grapefruit pulp. *J. Agric. Food Chem.* **1991**, *39*, 1075−1079.

(14) Javeri, H.; Wicker, L. Partial purification and characterization of peach pectinesterase. *J. Food Biochem.* **1991**, *15*, 241−252.

(15) Assis, S. A.; Lima, D. C.; Oliveira, O. M. M. F. Activity of pectinmethylesterase, pectin content and vitamin C in acerola fruit at various stages of fruit development. *Food Chem.* **2000**, *74*, 133−137.

(16) Körner, B.; Zimmerman, G.; Berk, Z. Orange pectinesterase: purification, properties and effects on cloud stability. *J. Food Sci.* **1980**, *45*, 1203.

(17) Kertesz, Z. I. Pectic enzymes. In *Methods of Enzymology*; Colowick, S. P., Kaplan, N. O, Eds.; Academic Press: New York, 1955; Vol. 1, pp 1581.

(18) Balaban, M. O.; Arreola, A. G.; Marshall, M.; Peplow, A.; Wei, C. I.; Cornell, J. Inactivation of pectinesterase in orange juice by supercritical carbon dioxide. *J. Food Sci.* **1991**, *56*, 743−746.

(19) Hartree, E. Determination of protein: A modification of the Lowry method that gives a linear phometric response. *Anal. Biochem.* **1972**, *48*, 422−427.

(20) Whitaker, J. R. Determination of the molecular weight of protein by gel filtration on Sephadex. *Anal. Chem.* **1963**, *35*, 1950−1953.

(21) Mcdonald, H. M.; Evans, R.; Spencer, W. J. Purification and properties of the major pectinesterase in lemon fruits (*Citrus limon*). *J. Sci. Food Agric.* **1993**, *62*, 163−168.

(22) Cameron, R. G.; Niedz, R. P.; Grohmann, K. Variable heat stability for multiple forms of pectinmethylesterase from citrus tissue culture cells. *J. Agric. Food Chem.* **1994**, *42*, 903−908.

(23) Cameron, R. G.; Grohmann, K. Partial purification and thermal characterization of pectinmethylesterase from red grapefruit finisher pulp. *J. Food Sci.* **1995**, *60*, 821−825.

(24) Pilnik W.; Voragen, A. G. J. The significance of endogenous and exogenous pectic enzymes in fruit and vegetable processing. In *Food Enzymology*; Fox, F. P., Ed.; Elsevier Applied Science: London, U.K., 1991; pp 7−333.

(25) Faye, L. Structure, biosynthesis, and function of plant glycoprotein glycans. In *Current Topics in Plant Biochemistry and Physiology*; Proceedings, Plant Biochemistry and Physiology Symposium; University of Missouri: Columbia, MO, 1988; pp 62−82.

(26) Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **1993**, *3*, 97−130.

(27) Cameron, R. G.; Grohmann, K. Purification and characterization of a thermally tolerant pectin methylesterase from a commercial fresh frozen orange juice. *J. Agric. Food Chem.* **1996**, *44*, 458−462.

(28) Nari, J.; Noat, G.; Ricard, J. Pectinmethylesterase, metal ions and plant cell wall extension. *Biochem. J.* **1991**, *279*, 343−350.

(29) Alonso, J.; Howell, N.; Canet, W. Purification and characterization of two pectinmethylesterase from persimmon (*Diospyros kaki*). *J. Sci. Food Agric.* **1997**, *75*, 352−358.

(30) Rexová-Benková, L.; Markovic, O. Pectic enzymes. *Adv. Carbohydr. Chem. Biochem.* **1976**, *33*, 323−385.

(31) Lee, M.; MacMillan, J. D. Mode de action of pectic enzymes. I. Purification and certain properties of tomato pectinesterase. *Biochemistry* **1970**, *9*, 1930−1934.

(32) Miller, L.; MacMillan, J. D. *Biochemistry* **1971**, *10*, 570−576.

(33) Gaffe, J.; Tieman, D. M.; Handa, A. K. Pectin methylesterase isoforms in tomato (*Lycopersicon esculentum*) tissues. *Plant Physiol.* **1994**, *105*, 199−203.

(34) Golderberg, R.; Pieron, M.; Durand, L.; Mutaftschiev, S. In vitro and in situ properties of cell wall pectin methylesterases from mung bean hypocotyls. *J. Exp. Bot.* **1992**, *43*, 41−46.

(35) Laats, M. M.; Grosdenis, F.; Recourt, K.; Voragen, A. G. J.; Wichers, H. J. Partial purification and characterization of pectin methylesterase from green beans (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **1997**, *45*, 572−577.