Purification and Properties of Pectin Methylesterase from Mandarin Orange Fruit

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Pectin methylesterase has been purified to homogeneity from mandarin orange according to a technique which employs an affinity chromatography step on heparin-Sepharose. The purified enzyme has a molecular weight of 37 000 and consists of a single polypeptide chain. Its K_m toward the citrus pectin is 0.84 mg/mL with a V_{max} of 0.38 μmol of acid produced/min. The enzyme exhibits a pH optimum around 9, and 50% inactivation occurred in 1 min at 62 °C. The protein is found to be glycosylated, and its amino acid composition has been reported.

INTRODUCTION

Pectin methylestrerase (PME) (EC 3.1.1.11) removes methoxyl groups from methylated pectic substances. This enzyme is widely distributed in plants, and the data published in the past few years have established that plants contain multiple forms of this enzyme (Hultin and Levine, 1963; Evans and McHale, 1978). Very little is known about the physiological role of these forms; they differ by molecular weight or have the same molecular weight but differ by charge. Recently, two PME forms differing in the glycosylation degree have been found in kiwi (Giovane et al., 1990). It seems that the different extent of glycosylation can affect the affinity for pectin and the thermostability of the enzyme forms. Technological investigations on PME isozymes from orange have been made with the aim to evaluate their kinetic and thermal behavior (Versteeg et al., 1978; Versteeg, 1980). In fact, the thermal inactivation of this enzyme is generally performed in industrial preparation of fruit juices to obtain juice stabilization (Joslyn and Pilnik, 1961). However, the finding of a proteic inhibitor of PME (Balestrieri et al., 1990) allowed its potential application in the stabilization of orange juice (Castaldo et al., 1992).

In the present paper we describe a method for PME purification that could be successfully employed for almost all of the fruit and vegetable species. Furthermore, we investigate some physicochemical properties of the purified enzyme that could be of general interest for the knowledge of the kinetic and thermal behavior of this enzyme.

MATERIALS AND METHODS

Materials. Citrus pectin was purchased from Sigma; its methylation degree was found to be 57% according to the method of Hinton (1940). DEAE-cellulose microgranular (DE-52) was from Whatman. Heparin-Sepharose CL-6B resin (45-165-μm particle size), Superose 12 prepacked column (10-μm particle size), and Sepharose 12 prep grade resin (30-μm particle size) were purchased from Pharmacia.

Protein Assay. The protein concentration was determined according to the method of Bradford (1976), using a calibration curve made with bovine serum albumin.

PME Activity Determination. PME activity was determined at 25 °C in a continuous spectrophotometric assay at pH 7.5 according to Hagerman and Austin (1986). This method was used in all of the determinations except the pH optimum assay. The incubation mixture was 3 mL; the pectin concentration was 0.2%. The activity units were expressed as micromoles of acid produced per minute in 1 mL of incubation reaction.

pH Optimum Determination. The pH dependence of mandarin orange PME was assayed titrimetrically in the pH range 3.5-11 at 25 °C according to the method of Vas et al. (1967). The reaction mixture was 5 mL and the pectin concentration 0.2%. The amount of PME was chosen to obtain a linear initial velocity over 5 min at the maximum activity. Blanks lacking PME were made for each determination, and the amount of acid produced, due to the spontaneous pectin demethylation, was subtracted.

Gel Electrophoresis Analysis. Gel electrophoresis in denaturing conditions was performed in sodium dodecyl sulfate (SDS-PAGE) according to the method of Laemmli (1970) on a Phast System (Pharmacia) in a gradient gel (10-15%). The molecular weight markers were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and lactalbumin (14 100).

Isoelectric point determination (p*I*) was performed on a Phast System apparatus (Pharmacia) in the pH range 3-9 using a calibration kit (Pharmacia) containing 10 proteins with isoelectric point ranging over 8.65-3.5.

The gel staining was performed with silver nitrate according to an automatic technique developed by Pharmacia based on the method of Heukeshoven and Dernik (1985).

Molecular Weight Determination. The molecular weight of the native PME was determined by gel filtration chromatography on a FPLC system (Pharmacia) equipped with a Superose 12 column HR10/30. The column was calibrated using the following proteins: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa). The column was eluted at flow rate of 0.5 mL/min with a buffer containing 10 mM Tris-HCl, pH 7.5, and 100 mM NaCl.

Thermal Stability. Each sample of PME (1 μg) in 20 μL of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM mercaptoethanol was placed by a microsyringe in a 400-μL plastic tube (4 × 45 mm) preheated for 5 min at each temperature tested and immediately capped. The sample was incubated for 1 min, cooled in ice, centrifuged, and assayed for activity.

Amino Acid Composition. Amino acid composition was determined by HPLC with a Pico Tag apparatus (Waters) by precolumn derivatization of the sample with phenyl isothiocyanate according to the method developed by Waters (Cohen et al., 1989). The protein hydrolysis was performed at 112-116 °C for 24 h in 200 μL of HCl (constant boiling) containing 0.5%

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Table I. Purification Steps of Pectin Methylesterase from Mandarin Orange

fraction	total vol, mL	total protein, mg	act., units	sp act., units/mg	yield, %	purifn, x-fold
crude extract	1000	1280	224 000	175	100	1
ammonium sulfate 0–80%	250	800	192 000	240	85.7	1.4
DEAE-cellulose	340	47.6	108 800	2285	48.6	13
heparin–Sepharose	3	1.9	80 641	42442	36	242
Superose 12	10	1.5	71 820	47 880	32	274

phenol. The vial was sealed under vacuum after three alternate vacuum nitrogen flushing steps.

PME Glycosylation Detection. Detection of sugars in PME was performed with a DIG glycan detection kit (Boehringer). The method is based on oxidation to aldehyde of hydroxyl groups in sugars linked to protein. The spacer linked hapten digoxigenin is then covalently attached to these aldehydes via a hydrazide group. Digoxigenin-labeled glycoconjugates are subsequently detected in an enzyme immunoassay using a digoxigenin-specific antibody conjugated with alkaline phosphatase. The samples were run in SDS–PAGE and then transferred onto a nitrocellulose sheet by electroblotting technique (Towbin et al., 1979) and assayed for glycosylation.

RESULTS AND DISCUSSION

The purification of pectin methylesterase (PME) from mandarin orange was achieved by utilizing a method developed on the basis of previous works on this enzyme (Castaldo et al., 1989a,b; Giovane et al., 1990) and consists of five steps.

First Step. The extract was obtained by homogenizing the fruits and centrifuging the suspension obtained. A solution of 1 M NaCl was added to the precipitate, and the suspension was stirred for 3 h at 4 °C, maintaining the pH at 8.5 by addition of 1 M NaOH. The suspension was centrifuged, and the supernatant was taken as crude extract (see Table I).

Second Step. Ammonium sulfate was added to the solution at a concentration of 80% saturation, and the precipitate, collected by centrifugation, was resuspended in 10 mM Tris-HCl, pH 7.5, and 20 mM NaCl and dialyzed against the same buffer.

Third Step. The dialyzed solution was loaded onto a DEAE-cellulose column (5 × 20 cm) equilibrated in the dialyzing buffer. The PME was unretained on this column; thus the column flow-through was collected, and the fractions containing activity were pooled.

Fourth Step. The pool obtained from the previous step was directly loaded onto a heparin–Sepharose column (2.5 × 10 cm) equilibrated in 10 mM Tris-HCl, pH 7.5, and 20 mM NaCl. The column was washed with the same buffer and the PME was eluted by a linear gradient from 20 to 300 mM NaCl. The fractions showing activity were pooled and concentrated by ultrafiltration.

Fifth Step. The concentrated pool was further purified by gel filtration chromatography on a FPLC system (Pharmacia) using a Superose 12 prep grade column (1.6 × 50 cm) by repeated injection of 500 μL of sample. The column was eluted at flow rate of 1 mL/min using a buffer containing 10 mM Tris-HCl, pH 7.5, and 100 mM NaCl. The fractions were analyzed by SDS–PAGE, and those containing the pure protein were pooled.

The major feature of this method was the affinity chromatography on the heparin–Sepharose column which had a good selectivity for PME; in fact, by this step, an enzyme of 90% purity was obtained. The kind of interaction between PME and heparin is probably due to a specific recognition of the heparin by PME because of the structural similarity between pectin and heparin (Castaldo et al., 1989a). From this point of view, the heparin–Sepharose chromatography can be performed as a general method for PME purification from different sources. Furthermore, the DEAE and heparin columns could be run in sequence without any further manipulation. The PME purification procedure is summarized in Table I. From 2 kg of fruits 1.5 mg of pure enzyme was obtained with a yield of 32%. In Figure 1 is shown a SDS–PAGE of the pools obtained from each step of purification. It should be noted that the PME from mandarin orange is purified as a single enzymatic form, whereas more than one form is generally described to be present in fruits (Castaldo et al., 1989b; Evans et al., 1978; Giovane et al., 1990; Versteeg et al., 1978). The SDS–PAGE analysis of the pure enzyme gave a molecular weight of 37 000, which was confirmed by gel filtration experiment on a Superose 12 column calibrated for molecular weight. This finding demonstrates that the mandarin orange PME consists of a single polypeptide chain. Isoelectrofocusing determination showed a p*I* value higher than 8.65. The uncertainty of p*I* determination is due to the PME focalization at the end of the gel; therefore, we can only conclude that PME has a p*I* higher than 8.65, which corresponds to the p*I* of lentil lectin, the most basic protein we utilized as standard. As far as the molecular weight and isoelectric point are concerned, the values found for mandarin orange PME are near those reported for this enzyme from orange (Versteeg et al., 1978).

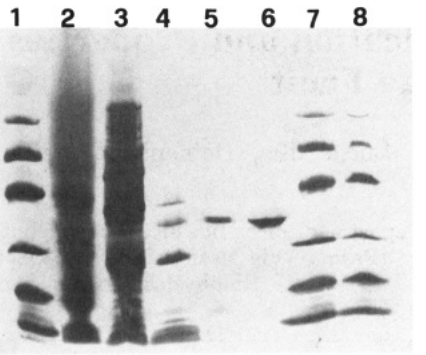

Figure 1. SDS–PAGE of pools from each purification step of PME. (Lanes 1, 7, 8) Molecular weight markers (see Materials and Methods); (lane 2) crude extract; (lane 3) ammonium sulfate; (lane 4) DEAE-cellulose; (lane 5) heparin–Sepharose; (lane 6) Superose 12.

Table II. Amino Acid Composition of PME from Mandarin Orange

amino acid	amt, g %	min residue no.	no. of residues/mol of protein[a]
Asp + Asn	9.61	13.37	27
Glu + Gln	8.75	11.01	22
Ser	6.84	12.05	24
Gly	5.88	14.50	29
His	0.93	1	2
Thr	8.60	13.37	27
Ala	8.47	17.61	35
Arg	7.50	7.98	16
Pro	0.71	1.15	2
Tyr	5.85	5.98	12
Val	6.66	10.52	21
Met	1.25	1.55	3
Cys	3.70	5.65	11
Ile	8.86	12.50	25
Leu	6.30	8.89	18
Phe	6.85	7.68	15
Lys	3.24	3.28	6

[a] Assuming a protein molecular weight of 37 000.

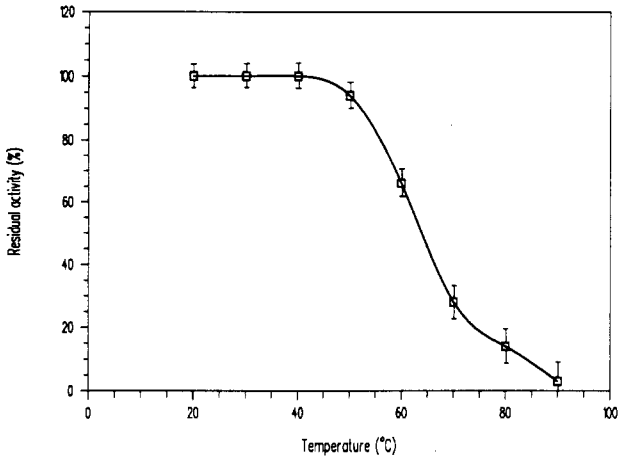

Figure 2. Thermal stability of PME. Each sample (in triplicate) was heated for 1 min at the indicated temperature.

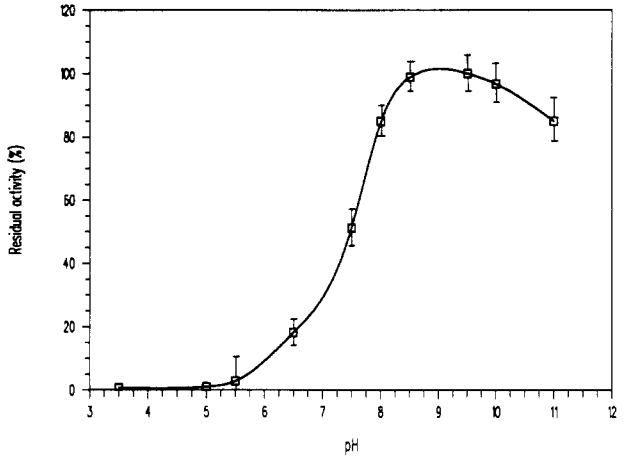

Figure 3. pH optimum determination of PME. Each sample (in triplicate) was assayed for activity at the indicated pH.

The K_m for citrus pectin was determined on the pure protein. The double-reciprocal plot was linear (correlation coefficient of 0.99) over 0.5–5 mg/mL pectin and gave a K_m value of 0.84 ± 0.08 mg/mL of pectin and a V_{max} of 0.38 ± 0.05 μmol of acid produced/min.

The PME thermal stability (Figure 2) was calculated by incubating the enzyme for 1 min at increasing temperature. The activity was substantially unaffected up to 50 °C and then it decreased to about 20% at 70 °C and was undetectable at 90 °C.

The effect of pH on PME activity is shown in Figure 3. The enzyme activity exhibited a maximum at pH 9 and was undetectable below pH 5.5. The pH optimum found for mandarin orange PME is considerably higher than that found for other PMEs, which generally is in the range 7–8.

The amino acid composition of PME was also determined as shown in Table II; moreover, a minimum molecular weight of 36 131 was calculated from the number of residues (Table II, second column). This value agrees with that found by gel filtration and electrophoresis techniques, considering also that the tryptophan is lost during the acid hydrolysis. The enzyme was found to be a glycoprotein by using an immunoassay detection kit (Boehringer); unfortunately, by this technique, it is not possible to assess the protein glycosylation degree. The finding, however, is not surprising; in fact, in other plant sources, PME and polygalacturonase were also found to be glycosylated (Moshrefi and Luh, 1983; Giovane et al., 1990).

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