Cloning and Expression of an Acidic Pectin Methylesterase from Jelly Fig (*Ficus awkeotsang*)

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Pectin methylesterase (PME) is the key enzyme responsible for the gelation of jelly curd in the water extract of jelly fig (*Ficus awkeotasang*) achenes. The jelly fig PME extracted from achenes was isoelectrofocused at pH 2.5 and subjected to N-terminal amino acid sequencing. A cDNA fragment encoding the mature protein of this acidic PME was obtained by PCR cloning using a poly(T) primer and a degenerate primer designed according to the N-terminal sequence of the purified PME. The complete cDNA sequence of its precursor protein was further obtained by PCR using the same strategy. The PME clone was overexpressed in *Escherichia coli*, and its expressed protein was immunologically recognized as strongly as the original antigen using antibodies against purified PME. Fractionation analysis revealed that the overexpressed PME was predominantly present in the pellet and thus presumably formed insoluble inclusion bodies in *E. coli* cells.

Keywords: *Gelation; isoelectrofocus; jelly fig; PCR cloning; pectin methylesterase*

INTRODUCTION

Jelly fig (*Ficus awkeotasang* Makino) is a unique woody vine growing in the mountainous areas of Taiwan. Gelation of the water extract from jelly fig achenes is extensively utilized to produce jelly curd for making a popular drink in local markets, particularly during the summer when the maturing jelly fig achenes are in season. Along with calcium ion and pectin in the water extract of the achenes, a unique pectin methylesterase (PME) (EC 3.1.1.11) has been identified as the key enzyme involved in the mechanism of gelation (Huang et al., 1980). The gelation forms a three-dimensional structure constructed via the ionic interaction between the calcium ion and the carboxyl group of pectin after demethoxylation by the unique PME.

PME, a ubiquitous enzyme in plants, de-esterifies the methoxylated pectin in the plant cell wall and is responsible for pectin degradation by the combined activities of polygalacturonase and pectate lyase (Gaffe et al., 1997). As a consequence of its enzymatic activity on pectin, PME has been regularly used in the wine, juice, and food industries (Gainvors et al., 1994; Giovane et al., 1996). It is generally assumed that PME plays an important role in plant cell growth and differentiation (Wen et al., 1999). Recently, a cell wall-associated PME was proposed to be a host cell receptor involved in cell-to-cell movement of tobacco mosaic virus (Dorokhov et al., 1999).

The PME responsible for gelation of the water extract from jelly fig achenes has been purified to apparent homogeneity and well-characterized (Lin et al., 1989). In contrast with the high isoelectric point (p*I*) of most known PMEs (around pH 9−11) (Bordenave et al., 1996), the jelly fig PME possesses a p*I* of pH 3.5 determined by isoelectric focusing. This acidic PME, a monomer with a molecular mass around 38 kDa estimated by SDS−PAGE and gel filtration, is heat-stable (up to 60 °C) and maintains its enzymatic activity over a broad pH range, particularly in the presence of sufficient ionic strength. In addition, its activity can be fully preserved after a long period (e.g., 6 months) of storage at 4 °C. The stability and enzymatic capability afford this acidic PME from jelly fig potential applications in industry (Komae et al., 1989; Lin et al., 1990). Direct purification of this enzyme from jelly fig achenes seems to be economically ineffective at this time. However, the potential applications may be feasible if the jelly fig PME can be easily produced using the available protein expression systems via genetic engineering. The corresponding gene of this acidic PME in jelly fig achenes has not been cloned so far.

In this study, we obtained a cDNA sequence from maturing jelly fig achenes by PCR cloning. The deduced amino acid sequence of this clone comprises the precursor protein of the acidic PME in jelly fig achenes with a calculated p*I* at pH 4.39 for its mature enzyme. The clone was further confirmed by immunological recognition on its overexpressed protein in *Escherichia coli* with antibodies against the purified enzyme.

MATERIALS AND METHODS

Plant Materials. Mature and fresh maturing jelly fig (*F. awkeotasang* Makino) achenes were obtained from a local grower. The mature achenes were used for purification of PME, and the fresh maturing achenes ~70 days after flowering were harvested for the construction of a cDNA library.

Preparation of Crude PME from Jelly Fig Achenes. Crude extract of jelly fig PME was prepared by soaking 50 g of jelly fig achenes in 500 mL of extraction buffer containing 4% NaCl and 2% polyvinylpolypyrrolidone (PVPP) for 24 h at room temperature (23 °C) according to the method described by Lin et al. (1989). Jelly fig PME released from the extracellular matrix of achenes was collected via filtration through six layers of cheesecloth and concentrated 200 times under nitrogen gas using an Amicon concentrator. The concentrated sample was subjected to isoelectrofocusing and SDS−PAGE.

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Isoelectrofocusing of PME. Isoelectrofocusing was performed in a Bio-Rad Rotofor cell using the procedure modified by Chuang et al. (1996). The horizontal cylindrical focusing cell of 55 mL was divided vertically into 20 chambers by partitions to minimize diffusion during electrophoresis and disturbance of the gradient during fractionation. Each partition was made of monofilament polyester screen of 6 μm × 6 μm pore size. Fractionation of the content in each chamber was achieved simultaneously within 1 s by a vacuum harvesting system. Jelly fig PME (130 μg) suspended in 1 mL of extraction buffer was mixed with the gradient solution containing 1% ampholyte (0.5% Bio-Lyte 6-8 and 0.5% Bio-Lyte 3-10, both from Bio-Rad). The sample was electrofocused in the pH gradient formed under an electric field at constant power (15 W) at 700 V for 100 min. After electrofocusing, the proteins fractionated in the 20 chambers were harvested. The protein content and pH along the gradient in these 20 fractions were detected at 280 nm with a spectrophotometer and a pH meter, respectively.

Analysis of Protein Contents in SDS−PAGE. Crude PME was resolved by glycine SDS−PAGE (Laemmli, 1970) using 12.5% polyacrylamide. The sample was extracted with the sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol according to the Bio-Rad instruction manual. Following electrophoresis, the gel was stained with Coomassie Blue R-250 or subjected to Western blotting for immunoassay.

Antibody Preparation and Western Blotting. Jelly fig PME resolved in SDS−PAGE was eluted according to a modified gel extraction procedure (Tzen et al., 1998) and subjected to induction of antibodies. Polyclonal antibodies against jelly fig PME were raised in chickens, and immunoglobulins were purified from egg yolks for the immunoassays (Polson, 1990). In the immunoassays, proteins resolved in the SDS−PAGE gel were transferred onto a nitrocellulose membrane in a Bio-Rad Trans-Blot system according to the manufacturer's instructions. The membrane was subjected to immunodetection using secondary antibodies conjugated with horseradish peroxidase (Sigma) and then incubated with 4-chloro-1-naphthol containing H_2O_2 for color development as described by Tai et al. (1999).

Partial Amino Acid Sequencing. PME eluted from the SDS−PAGE gel was transferred onto a piece of PVDF membrane (Immobilon-P transfer membrane purchased from Millipore) and then subjected to N-terminal sequencing by the Applied Biosystems 476A protein sequencer at Chung-Hsing University, Taiwan, using the procedure described by Chen et al. (1999).

Isolation of Total RNA and Poly(A)$^+$ RNA. Total RNA was extracted from the maturing achenes of jelly fig ~70 days after flowering ground in liquid nitrogen using the phenol/SDS method (Wilkins and Smart, 1996). Poly(A)$^+$ RNA was isolated with Dynabeads (Dynal) following the manufacturer's instructions. The isolated poly(A)$^+$ RNA was dissolved in DEPC-treated water and then quantitated as the absorbance at 260 nm with a spectrophotometer.

cDNA Library Construction, Screening, and Sequencing. cDNA was synthesized from poly(A)$^+$ RNA according to the protocol described in the manufacturer's instructions (cDNA synthesis, ZAP-cDNA synthesis, and ZAP-cDNA Gigapack III Gold Cloning kits purchased from Stratagene). A cDNA library of ~10^6 plaques was constructed with 10 μg of poly(A)$^+$ RNA. The plaques were subjected to in vivo excision of the pBluescript phagemid from the Uni-ZAP XR vector following the manufacturer's instructions. A degenerate primer (5′-ATHGARCCNGAYATNGTNGTNGC-3′) was designed according to the first eight residues of the N-terminal amino acid sequence of purified PME. PCR amplification was carried out using the designed primer and a poly(T) primer with the excised phagemids as templates. A PCR fragment of ~1300 bp was harvested, ligated into the pGEM-T Easy Vector systems (Promega), and subjected to sequencing using the Sequence Version 2.0 DNA sequence kit (USB). To obtain the complete cDNA encoding the precursor protein (including presequence and mature enzyme) of PME, a primer was designed according to the sequence corresponding to the middle region of mature enzyme. Under the same strategy of PCR cloning using the designed primer and a vector primer, a DNA fragment of ~1500 bp was obtained and sequenced.

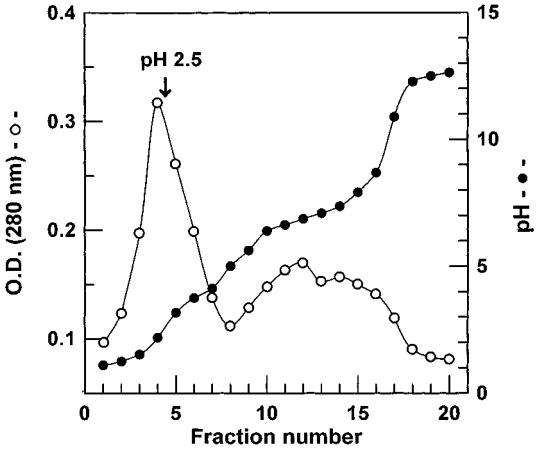

Figure 1. Isoelectrofocusing of the acidic PME in jelly fig achenes. Isoelectrofocusing was performed in a horizontal Bio-Rad Rotofor cell. The pH gradient was formed under an electric field. After electrofocusing, the proteins in the pH gradient were fractionated into 20 tubes. The distribution of proteins detected at OD 280 nm (open circles) and the pH gradient (solid circles) were recorded for each tube. An arrow indicates the position where the sample was focused.

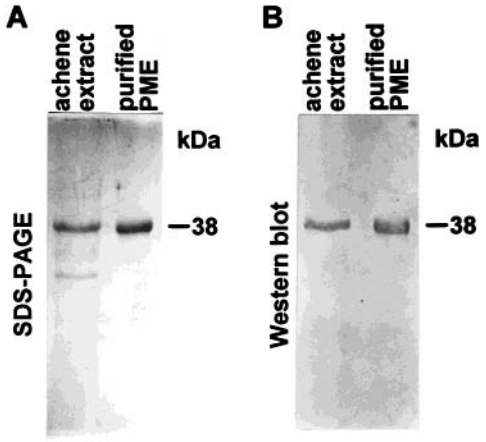

Figure 2. SDS−PAGE and Western blotting of the PME purified from jelly fig achenes: (A) jelly fig PME extracted from the extracellular matrix of achenes or further purified by isoelectrofocusing resolved in SDS−PAGE; (B) duplicate gel with half of the protein content transferred onto nitrocellulose membrane and then subjected to immunoblotting using antibodies against the purified PME. Labels on the right indicate the molecular mass of the jelly fig PME.

Sequence Analyses and Prediction. Sequence comparisons were performed with GenBank using the Blast program (Altschul et al., 1990). N-terminal signal sequence was predicted using the SignalP program in the World Wide Web Prediction Server Center for Biological Sequence Analysis (Nielsen et al., 1997). PME motif was identified using the Motif program at GenomeNet, Japan (www.motif.genome.ad.jp). The O-glycosylation site was predicted using the NetOGlyc 2.0 program at the CBS Prediction Server (www.cbs.dtu.dk). The N-glycosylation site was predicted using the ScanProsite program at the ExPASy Molecular Biology Server (www.expasy.ch/tools/scnpsit1.html). The transmembrane segment was analyzed using the SOSUI program designed by the Itaku group at the Department of Biotechnology, Tokyo University of Agriculture and Technology (azusa.proteome.bio.tuat.ac.jp/sosui).

Overexpression of the Jelly Fig PME Clone in *E. coli*. The cDNA clone of jelly fig PME was constructed either in the

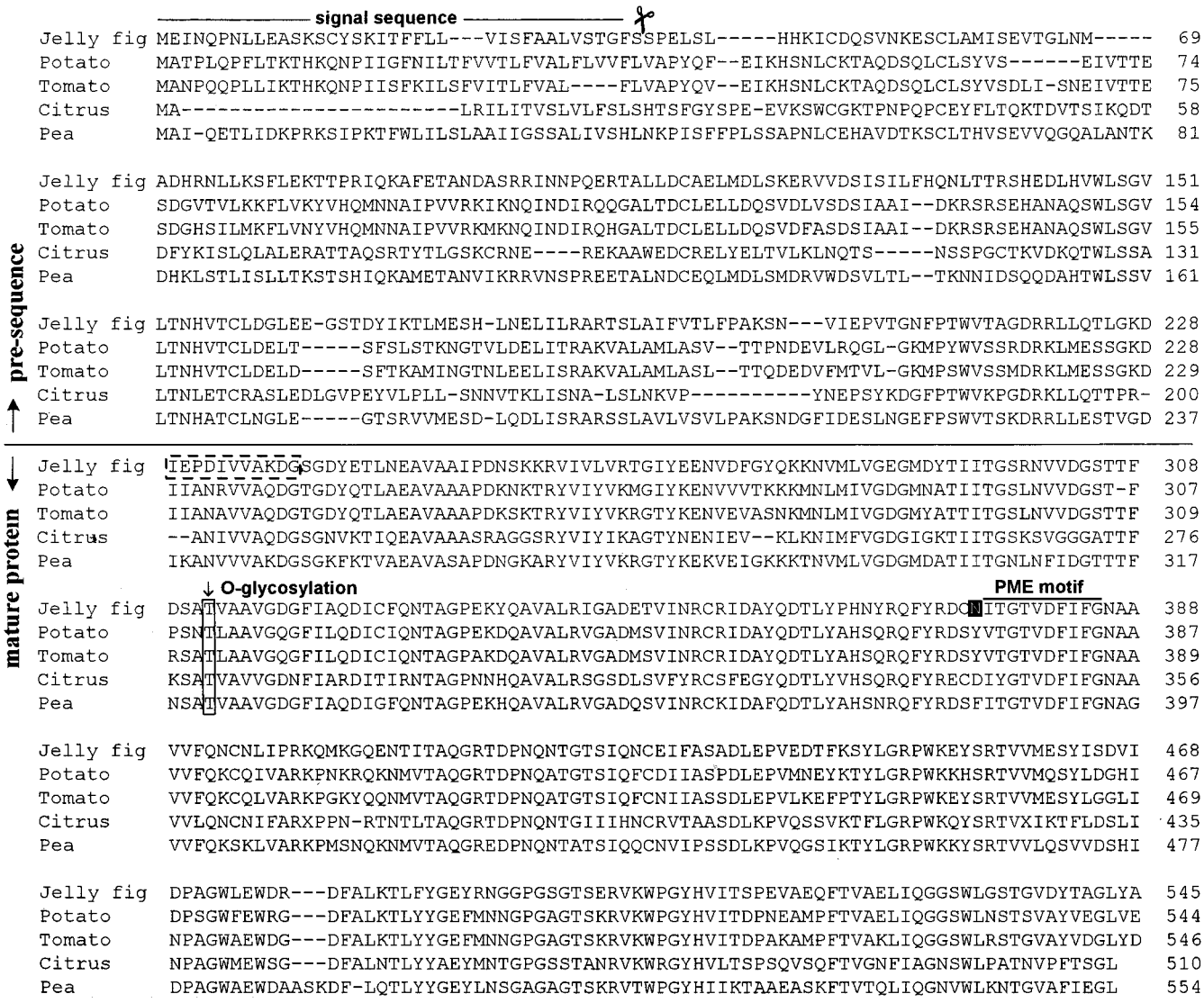

Figure 3. Sequence alignment of the precursor protein of jelly fig PME with other homologous sequences. The amino acid number for the last residue in each line is listed on the right for each species. Broken lines in the sequences represent gaps introduced for best alignment. The cleavage site of the putative N-terminal signal sequence is indicated by a scissors symbol. The first 11 N-terminal residues of mature jelly fig PME obtained directly from amino acid sequencing are enclosed with dotted lines. A unique O-linked glycosylation site is boxed and indicated by an arrow. A potential N-linked glycosylation site in jelly fig PME is highlighted. The conserved PME motif is indicated on top of the sequences. The accession numbers of the aligned sequences are as follows: potato, Q96576; tomato, P14280; citrus, U82977; pea, X67425.

nonfusion expression vector, pET29a(+), or in the fusion expression vector, pET28a(+) (Novagen), using an *Nde*I site at the initial methionine position and an *Xho*I site in the polylinker of the vectors. An initial methionine was affixed to the expressed PME in the nonfusion expression. The recombinant fusion protein comprised an N-terminal appendix of 20 amino acid residues (MGSSHHHHHHSSG LVPRGSH). The recombinant plasmid was used to transform *E. coli* strain BL21 (DE3). Overexpression was induced by 1 mM IPTG in a bacteriophage T7 RNA polymerase/promoter system. At various time intervals (0.5, 1, 2, and 3 h) after induction, the *E. coli* cells were harvested, fractionated into supernatant and pellet by centrifugation, lysed by heating at 95 °C for 10 min with the sample buffer, and then subjected to SDS−PAGE and Western blot analyses.

RESULTS

Isoelectrofocusing of an Acidic PME Extracted from Jelly Fig Achenes. Crude extract of jelly fig PME released from the extracellular matrix of achenes was subjected to isoelectrofocusing using the Bio-Rad Rotofor cell system. After electrofocusing, a major protein peak was detected at pH 2.5 (Figure 1). The major protein was collected, concentrated, and then subjected to analysis by SDS−PAGE (Figure 2A). The result indicates that the acidic protein obtained from isoelectrofocusing is the 38 kDa PME of jelly fig achenes as reported previously by Lin et al. (1989). Antibodies against this acidic PME were successfully generated in chickens as detected by immunodetection (Figure 2B).

Cloning of a cDNA Sequence Encoding the Acidic PME from Maturing Achenes of Jelly Fig. To clone the corresponding gene of the acidic PME in jelly fig, the first 11 amino acid residues (IEPDIVVAKDG, counting from the N terminus) of the purified PME were determined by direct sequencing and used to design a degenerate primer. A complete cDNA sequence encoding the precursor protein of this acidic PME was obtained by PCR cloning. Aligned with homologous PME in other species, the deduced amino acid sequence of the clone consists of an N-terminal

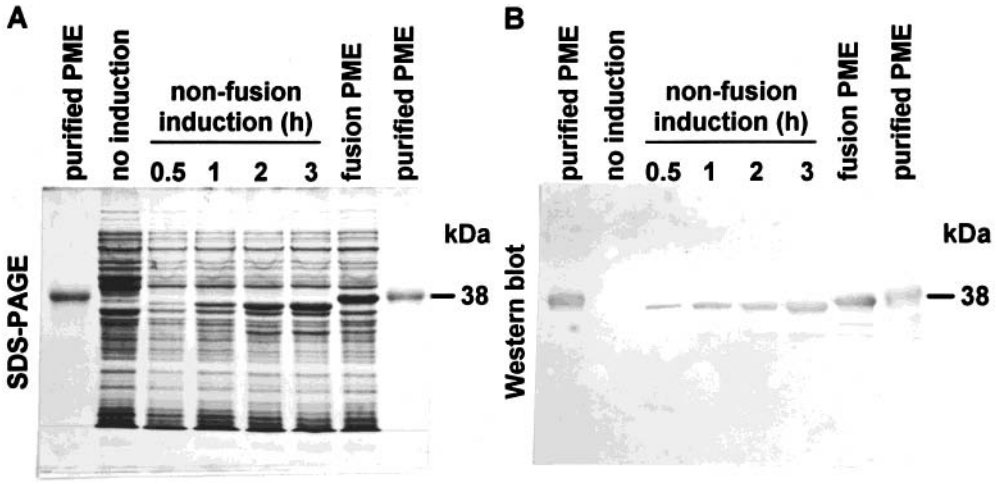

Figure 4. SDS−PAGE and Western blotting of the *E. coli* cells containing the overexpressed jelly fig PME: (A) jelly fig PME purified from achenes (2 μg) or overexpressed in *E. coli* cells (10 μg total proteins) at various time intervals after induction resolved in SDS−PAGE (the fusion protein containing jelly fig PME was harvested 3 h after induction); (B) duplicate gel with half of the protein content transferred onto nitrocellulose membrane and then subjected to immunoblotting using antibodies against the purified PME. Labels on the right indicate the molecular mass of the jelly fig PME.

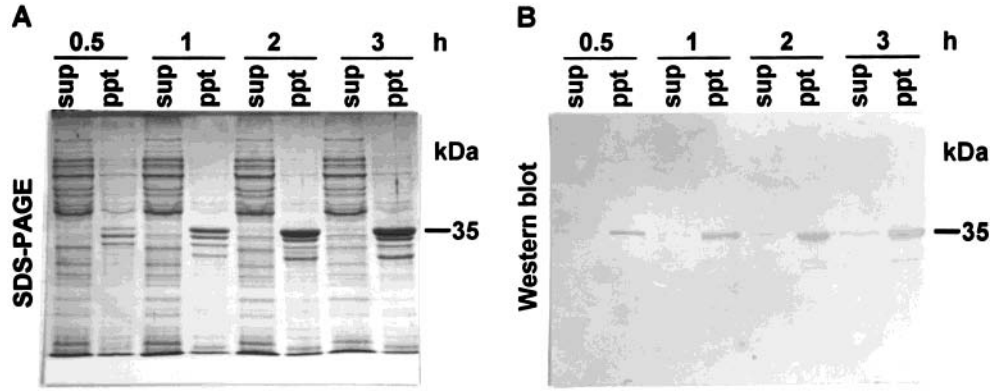

Figure 5. SDS−PAGE and Western blotting of the fractionated supernatant and pellet of the *E. coli* containing the overexpressed jelly fig PME: (A) supernatant (sup) and pellet (ppt) fractions of *E. coli* lysate (10 μg total proteins) containing the overexpressed jelly fig PME at various time intervals after induction resolved in SDS−PAGE; (B) duplicate gel with half of the protein content transferred onto nitrocellulose membrane and then subjected to immunoblotting using antibodies against the purified PME. Labels on the right indicate the molecular mass of the overexpressed jelly fig PME.

presequence and the mature enzyme of the jelly fig PME (Figure 3). Both length and sequence of mature enzyme are much more conserved than those of the presequence among diverse species. The presequence of jelly fig PME comprises 228 residues with a cleavable signal sequence for targeting to endoplasmic reticulum (ER) and is putatively responsible for transporting the mature protein to the extracellular matrix. The mature jelly fig PME comprises 317 residues with a conserved PME motif (residues 376−385) and a putative O-linked glycosylation site at Thr312. A potential N-linked glycosylation site is found at Asn375 of jelly fig PME but not present in other homologous genes. The protein is relatively hydrophilic and presumably contains no transmembrane segment. The deduced molecular mass of mature jelly fig PME without post-translational modification is 35053 Da, and the calculated p*I* is pH 4.39. The amino acid composition of the jelly fig PME deduced from the cDNA clone (Table 1) is in good agreement with that chemically determined by Lin et al. (1989). It is assumed that the cloned PME cDNA sequence encodes the acidic PME in the water extract of jelly fig achenes.

Immunodetection of Overexpressed PME in *E. coli*. The cDNA clone of jelly fig PME was constructed in a fusion or nonfusion vector and then overexpressed in *E. coli*. The jelly fig PME overexpressed in *E. coli* at various time intervals after induction was resolved by SDS−PAGE (Figure 4A) and further detected by im-

Table 1. Comparison of Amino Acid Composition of Jelly Fig PME As Determined by Chemical Analysis (Lin et al., 1989) or Deduced from Its Corresponding Gene

	amino acid composition of PME (mol %)	
	chemical analysis	deduced from PME clone
Asx	15.3	12.9
Glx	10.9	10.7
Ser	5.0	5.0
Gly	10.1	9.5
His	0.8	0.6
Arg	4.5	4.7
Thr	6.9	7.9
Ala	8.8	7.6
Pro	4.1	3.8
Tyr	5.9	5.4
Val	7.1	8.5
Met	1.3	1.3
Ile	5.5	6.6
Leu	4.8	4.4
Phe	5.1	4.1
Lys	3.7	3.8
Cys		1.6
Trp		1.6

munoassay using antibodies raised against purified PME (Figure 4B). The result reveals that the expressed fusion and nonfusion proteins containing jelly fig PME were recognized as strongly as the original antigen. The molecular mass (35 kDa) of the overexpressed jelly fig PME from the nonfusion expression is 3 kDa less than that (38 kDa) of purified PME. Inclusion of an N-terminal appendix of 20 residues (2.2 kDa) to jelly fig PME in the fusion expression shifted the migration of the recombinant fusion protein (37 kDa) to a location close to that of purified PME. The overexpressed jelly fig PME was predominantly present in the insoluble pellet of *E. coli* lysate harvested at various time intervals after induction (Figure 5). Similar results were obtained in the centrifuged fractions of the expressed fusion protein containing jelly fig PME in *E. coli* lysate (data not shown). It is likely that the overexpressed jelly fig PME formed inclusion bodies in *E. coli* as early as its initial accumulation after induction.

DISCUSSION

In this study, a cDNA sequence encoding the acidic PME responsible for the gelation of the water extract of jelly fig achenes was successfully cloned. The calculated low p*I* (pH 4.39) of this acidic enzyme is consistent with the observed p*I* in isoelectrofocusing (Figure 1; Lin et al., 1989). The successful cloning of this acidic PME from jelly fig enhances the potential for applying this unique enzyme in the wine, juice, and food industries. We are attempting to express this acidic PME in yeast or plant tissues that can be supplemented directly to the processing materials used in the above industries.

The molecular mass of the overexpressed PME estimated in SDS−PAGE, 35 kDa (Figure 4), is in accord with that calculated from the deduced amino acid sequence of the PME clone. However, the molecular mass of PME purified from jelly fig achenes is 38 kDa, estimated in SDS−PAGE (Figure 4) and gel filtration (Lin et al., 1989). The discrepancy may result from glycosylation of this cell wall enzyme in a similar pattern as reported in an extracellular glycosylated PME of *Clostridium thermosaccharolyticum* that contains 10% (w/w) carbohydrates in its mature protein (Van Rijssel et al., 1993). It remains to be investigated what kinds of carbohydrates are present in the jelly fig PME and what the biological function of the glycosylation is in this acidic enzyme.

According to the analysis of its amino acid sequence, the jelly fig PME is a relatively hydrophilic protein composed of abundant negatively charged residues. The overexpressed fusion protein containing this acidic PME was presumably assembled to form insoluble inclusion bodies early in its accumulation in *E. coli* (Figure 5). The insoluble overexpressed PME could be solublized with 7 M urea followed by dialysis; however, the solublized protein lacked detectable enzymatic activity (data not shown). Whether the loss of enzymatic activity in the solublized PME resulted from a misfolding of protein structure or a lack of glycosylation in the *E. coli* expression remains to be elucidated.

ABBREVIATIONS USED

p*I*, isoelectric point; PME, pectin methylesterase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, Polymerase Chain Reaction.

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