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Purification and Glycosylation Analysis of an Acidic Pectin Methylesterase in Jelly Fig (*Ficus awkeotsang*) Achenes

JOE L. C. DING, JOYCE S. F. HSU, MIKI M. C. WANG, AND JASON T. C. TZEN*

Graduate Institute of Agricultural Biotechnology, National Chung-Hsing University, Taichung, Taiwan 40227 ROC

An acidic pectin methylesterase (PME) is responsible for the gelation of water extract from jelly fig (*Ficus awkeotasang*) achenes. A new, fast and efficient, method has been developed to purify this acidic PME. The method includes preparing jelly curd by traditional hand washing, extracting proteins from the curd, and separating PME by anion-exchanger. The purified PME exists as a monomer of 38 kDa determined by gel filtration, and exerts enzymatic activity over a broad pH range, particularly in acidic environments where most known PME enzymes from various species are inactivated. Chemical staining and enzymatic cleavage suggest that the jelly fig PME is an N-linked glycoprotein. Fluorophore-assisted carbohydrate electrophoresis reveals that the polysaccharide of this glycoprotein putatively consists of 22 hexoses including 16 mannose, 4 *N*-acetylglucosamine, and 2 galactose residues.

KEYWORDS: Gelation; jelly fig; N-glycosylation; pectin methylesterase; polysaccharide

INTRODUCTION

Pectin methylesterase (PME) (EC 3.1.1.11), a ubiquitous enzyme in plants, de-esterifies the methoxylated pectin in plant cell wall and is responsible for pectin degradation with the combined activities of polygalacturonase and pectate lyase (*1*). It is generally assumed that PME plays an important role in plant cell growth and differentiation (*2*). Recently, a cell-wall-associated PME was proposed to be a host cell receptor involved in cell-to-cell movement of tobacco mosaic virus (*3, 4*).

Owing to its enzymatic activity on pectin, PME has been constantly used in the wine, juice, and food industries (*5, 6*). An acidic PME purified from jelly fig (*Ficus awkeotasang* Makino) achenes was characterized to be heat-stable (up to 60 °C) and enzymatically active over a broad pH range, particularly in the presence of sufficient ionic strength (*7*). In addition, its activity can be fully preserved after a long period (e.g., six months) of storage at 4 °C. The thermostability and enzymatic capability of this acidic PME elevate its potential applications in industry (*8, 9*).

The acidic PME has been identified as the key enzyme responsible for the gelation of water extract from jelly fig achenes. The gelled product, jelly curd, is utilized for preparation of a popular drink in Taiwan (*10*). The gelation forms a three-dimensional structure constructed via the ionic interaction between calcium ion and the exposed carboxyl groups of pectin after demethoxylation by the coexistent PME. Current protocol for purification of jelly fig PME from achenes is time-consuming (more than 3 days) and economically ineffective. Applications of jelly fig PME in industries would become feasible if this enzyme can be easily purified with high yield or produced by well-established expression systems via genetic engineering.

Glycosylation of PME has been reported in several species including kiwi (*11*), orange (*12*), and filamentous fungus (*13*). Two PME isoforms with different glycosylation have been found in kiwi (*11*), and the difference in glycosylation seems to affect their thermostability and pectin affinity. Similar observation on the effect of different N-glycosylation was reported for orange PME isoforms (*14*).

Recently, a cDNA sequence encoding the acidic PME of jelly fig was obtained by PCR cloning (*15*). The overexpressed PME in *Escherichia coli* (35 kDa) is smaller than the native PME (38 kDa), and lacks detectable enzymatic activity. Whether these observations are related to the glycosylation of native enzyme needs to be clarified.

In this study, we developed a new method to purify the acidic PME from jelly curd. The whole process could be completed in a few hours with good recovery yield. The PME was chemically and enzymatically determined as an N-linked glycoprotein, and its monosaccharide composition was analyzed.

MATERIALS AND METHODS

Extraction of PME from Jelly Curd. Jelly fig (*Ficus awkeotasang* Makino) achenes were purchased from local growers. Jelly curd was prepared by hand-washing 50 g of achenes wrapped in six layers of cheesecloth using 600 mL of extraction buffer containing 0.6 M NaCl, 2% polyvinylpolypyrrolidone (PVPP), and 10 mM Tris−HCl, pH 7.5. The extract was kept immobile for gelation at room temperature for 30 min. After gelation, the jelly curd containing PME was homogenized with 600 mL of the extraction buffer using a Polytron PT3000 (Kinemaica Ag) at 7000 rpm for 30 s. The insoluble pellet containing mostly pectin polymers was removed by centrifugation at 15000*g* for

* Corresponding author. Phone: 886-4-22840328. Fax: 886-4-22853527. E-mail: TCTZEN@dragon.nchu.edu.tw.

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10 min. Extracted PME in the soluble supernatant was precipitated with ammonium sulfate at a final concentration of 85% saturation and collected by centrifugation at 15000*g* for 30 min after stirring at room temperature for 10 min. The precipitate was suspended in 10 mL of minimal buffer (10 mM Tris−HCl, pH 7.5), and then dialyzed against 1 L of minimal buffer supplemented with 0.15 M NaCl at 4 °C for 30 min.

Separation of PME by DEAE Sephadex. The dialyzed sample was mixed with 10 mL of minimal buffer and applied to an anionic DEAE-Sephadex A-25 column (Pharmacia) previously equilibrated with minimal buffer supplemented with 0.15 M NaCl. After sample absorption, PME was eluted with 320 mL of a linear gradient of 0.15 to 0.6 M NaCl in minimal buffer at a flow rate of 1 mL min^{-1}. Protein profile of the column eluent was monitored at 280 nm. The eluent was concentrated by Amicon concentrator (Amicon) to 1.5 mL, and then subjected to enzymatic activity assay.

Spectrophotometric Assay of PME Enzymatic Activity. PME activity was determined using a continuous spectrophotometric assay at 30 °C according to the method described by Hagerman and Austin (*16*). The assay comprised 0.2% apple pectin (purchased from Sigma, approximately 80% methoxylated) in 3 mL of reaction mixture, and activity was expressed as μmol of acid produced per min in 1 mL of incubation reaction.

Analysis of Protein Contents by SDS−PAGE. Protein samples were resolved by SDS−PAGE (*17*) using 12.5% acrylamide. The samples were 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.

Protein Assay. Protein sample was mixed with a reaction reagent containing 2% sodium carbonate, 0.02% sodium tartrate, and 0.01% cupric sulfate for 20 min at room temperature. The mixture was further reacted with Folin−Ciocalteu's phenol reagent (Sigma) for 30 min. By reading sample absorption at 500 nm, the protein content was calculated from a linear standard equation derived from the absorption readings of a serial dilution of known bovine serum albumin concentrations.

Determination of Molecular Mass of Purified PME by Gel Filtration. The molecular mass of native PME was calculated by gel filtration chromatography using a Sephadex G-75 column calibrated with standard proteins, bovine serum albumin (67 kDa), ovalbumin (43 kDa), and soybean trypsin inhibitor (20.1 kDa). Purified PME was applied onto the column, which was preequilibrated with minimal buffer supplemented with 0.15 M NaCl, and then eluted at a flow rate of 0.5 mL min^{-1} using the same buffer. Protein profile of the column eluent was monitored at 280 nm. Enzymatic activity of PME was detected by the spectrophotometric assay as described previously.

Gel Activity Staining of PME. The enzymatic activity of PME was analyzed by nondenaturing gel electrophoresis containing 12.5% acrylamide and 0.1% apple pectin. The electrophoresis was performed at 4 °C for 5 h at a constant voltage (50 V). After electrophoresis, the gel was stained with Coomassie blue R-250 or immersed in 0.1 M phosphate buffer (pH 3, 4.5, 6, 7.5, or 9) prior to activity staining with 0.02% rethenium red in the minimal buffer. To evaluate the relative activity of PME at various pH environments, equal PME amounts were applied to each buffer solution and stained in the same condition.

Detection of Glycosylation in PME. Purified PME was denatured in a solution of 1% SDS and 5 mM β-mercaptoethanol for 5 min at 100 °C. Subsequently, the denatured PME was resolved by SDS−PAGE and subjected to staining of carbohydrate using an improved periodic acid-Schiff (PAS) reaction (*18*). To examine whether the attached carbohydrate is N-glycosylated to the protein, 10 μg of the above denatured PME was treated with 10 mU PNGase F (Sigma) at 37 °C for 24 h in a buffer containing 50 mM EDTA, 0.02% (w/v) sodium azide, and 20 mM sodium phosphate, pH 7.2. The change in molecular mass of PME before and after PNGase F digestion was analyzed in SDS−PAGE.

Overexpression of Jelly Fig PME in *E. coli*. The cDNA clone of jelly fig PME was constructed in the nonfusion expression vector, pET29a(+) (Novagen) as reported previously (*15*). The expressed PME comprised the entire sequence of mature jelly fig PME without presequence. Overexpression was induced by 1 mM IPTG in a bacteriophage T7 RNA polymerase/promoter system. The *E. coli* cells were harvested 3 h after induction, lysed by heating at 95 °C for 10 min with the sample buffer, and then subjected to SDS−PAGE analysis.

Western Blotting. Polyclonal antibodies against jelly fig PME were raised in chicken previously (*15*). In the immunoassays, proteins resolved in the SDS−PAGE gel were transferred onto 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. (*19*).

Fluorophore-Assisted Carbohydrate Analysis. The monosaccharide composition of jelly fig PME was determined using fluorophore-assisted carbohydrate electrophoresis (FACE) according to the method described by Jackson (*20*). Jelly fig PME was first purified by concanavalin A affinity column (Pharmacia) and eluted by α-D-methyl-mannoside. Eluted jelly fig PME of 32 μg was subjected to hydrolysis reaction containing 4 M trifluoroacetic acid at 100 °C for 5 h to release free monosaccharides. The released monosaccharides along with standard neutral and amino sugars including D-galactose, L-fucose, D-mannose, *N*-acetylgalactosamine, and *N*-acetylglucosamine (all purchased from Sigma) were fluorescence-labeled with 2-aminoacridone (Glyko). In the labeling reaction, each carbohydrate was suspended in 5 μL of reaction solution containing 0.1 M 2-aminoacridone (prepared in 3:17 acetic acid/water) and 1 M sodium cyanoborohydride (freshly prepared in dimethyl sulfoxide), and incubated at 37 °C for 15 h (*21*). After labeling, the samples were dried under nitrogen at 45 °C, mixed with 50 μL of loading buffer (20% glycerol in 62.5 mM Tris−HCl, pH 6.8), and then subjected to gel electrophoresis. The gel consisted of a 20−40% acrylamide linear gradient in a 14 × 16 × 0.75 mm^3 glass cassette. The gel buffer and the running buffer contained 0.42 M Tris base−borate (pH 8.5) and 0.1 M Tris base−borate/boric acid (pH 8.3), respectively. Electrophoresis was performed at a constant current of 40 mA for 2.5 h in a 4 °C cold room. After electrophoresis, the gels were imaged using a fluorescence camera linked to the gel system. The imaged bands of the released monosaccharides from PME were assigned by comparing with those of the standard sugars. Intensity of the imaged bands was quantitated by TINA software (version 2.09e).

RESULTS

Purification of PME from Jelly Curd. Jelly curd was prepared from the extract of jelly fig achenes by the traditional hand washing method used in Taiwan local markets. Both pectin and PME were extracted from the intercellular matrix of jelly fig achenes using a high salt condition (0.6 M NaCl). The extracted pectin was demethoxylated by the coexistent PME and allowed for gelation with calcium ion at room temperature. After gelation, PME was separated from pectin by extracting the jelly curd via homogenization. The crude PME was concentrated by ammonium sulfate precipitation and subjected to DEAE column chromatography (**Figure 1**). After absorption onto the column, jelly fig PME was eluted with a salt gradient of 0.15−0.6 M NaCl. High purity of jelly fig PME was obtained after DEAE chromatography (**Figure 2**). As revealed in SDS−PAGE, not many proteins are present in the extract of jelly curd, and PME is apparently the most abundant protein.

The whole process of purification was achieved in a few hours with good recovery yield (**Table 1**). In our preparation, 27.3 mg of PME could be obtained from 50 g of jelly fig achenes consistently. The recovery yield of this new method is higher than that (18.3 mg PME from 50 g achenes) of a previous protocol developed by Lin et al. (*9*). The molecular mass of jelly fig PME estimated from Sephadex G-75 column was 38 kDa (**Figure 3**). Presumably, the extracted PME exerted its enzymatic activity as a monomer.

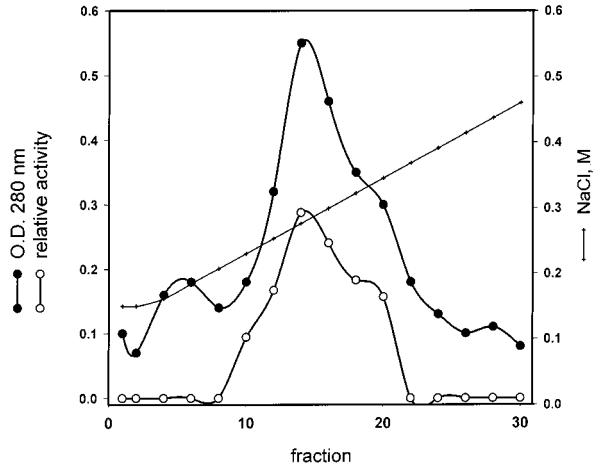

Figure 1. DEAE-Sephadex A-25 column chromatography of jelly fig PME extracted from jelly curd. Proteins were eluted with a salt gradient of 0.15–0.6 M NaCl. Protein profile (●) and PME activity (○) were separately monitored as described in Materials and Methods.

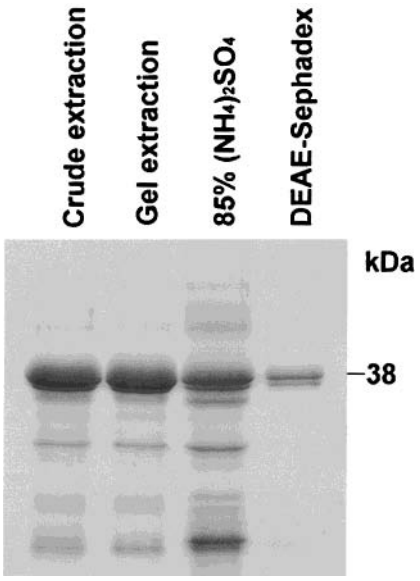

Figure 2. SDS–PAGE of proteins in PME preparation at different steps of purification. To compare the relative purity and recovery yield of PME at different steps of purification, the loaded protein amount of each sample was adjusted to represent the content extracted from an equal quantity of jelly fig achenes. Label on the right indicates the molecular mass of jelly fig PME.

Table 1. Purification of PME from Jelly Fig Achenes (50 g)

fraction	total protein (mg)	activity (units)	specific activity (units mg^{-1})	recovery (%)
crude extraction	279	13791	49	100
gel extraction	276	13534	49	98
85% $(NH_4)_2SO_4$	210	10796	51	78
DEAE-Sephadex	27.3	7731	283	56

Broad pH Range of Enzymatic Activity of Jelly Fig PME. A gel activity staining was designed to examine PME capability in various pH conditions. In this method, purified jelly fig PME was resolved in a nondenaturing gel and then subjected to activity staining by incubating the gel with pectin and dye (rethenium red) in various pH buffer solutions (**Figure 4**). The results indicated that jelly fig PME exerted enzymatic activity over a broad pH range, particularly in the low pH conditions

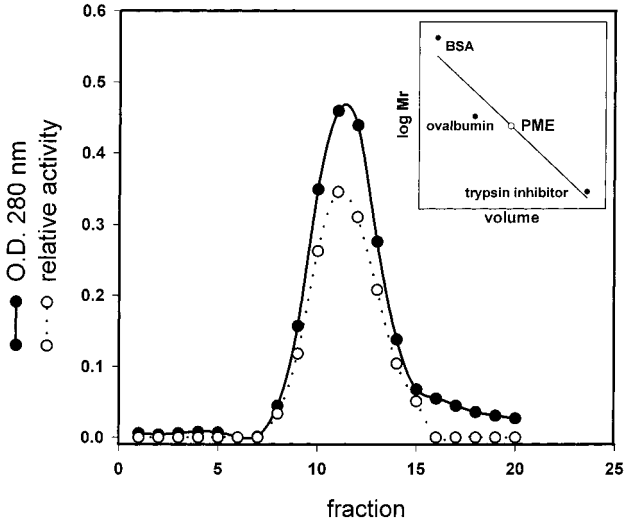

Figure 3. Gel filtration (Sephadex G75) column chromatography of purified PME. Protein profile (●) and PME activity (○) were separately monitored as described in Materials and Methods. The molecular mass of PME was calibrated using three marker proteins: bovine serum albumin (BSA, 67 kDa), ovalbumin (43 kDa), and soybean trypsin inhibitor (20.1 kDa) as shown in the inserted panel at the top right.

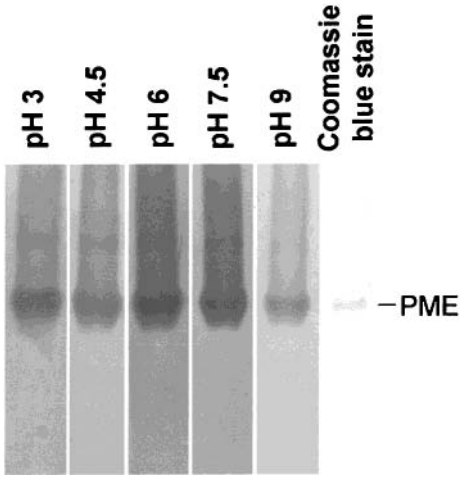

Figure 4. Gel activity staining of purified PME in various pH conditions. Purified jelly fig PME was resolved in a nondenaturing gel and then stained with Coomassie blue or subjected to enzymatic activity staining using 0.02% rethenium red at pH 3, 4.5, 6, 7.5, or 9.

where most known PME enzymes from various species are inactivated. Meanwhile, a few PME molecules seem to aggregate in our preparation, as minor bands are found in the upper region of the activity staining.

N-Glycosylation of Jelly Fig PME. To determine whether jelly fig PME is a glycoprotein, purified PME was resolved in SDS−PAGE (**Figure 5A**) and stained for carbohydrate by an improved PAS reaction (**Figure 5B**). Similar to hen egg immunoglobulin IgG, a known glycoprotein, jelly fig PME (38 kDa) purified from jelly fig achenes was positively stained in the reaction. However, the overexpressed PME (35 kDa) in *E. coli* could not be stained in the same condition despite its equivalent recognition by antibodies raised against the purified PME (**Figure 5C**). These results indicate that the PME purified from jelly fig achenes is glycosylated, whereas the overexpressed PME in *E. coli* is not.

To test if PME is an N-linked glycoprotein, the SDS-denatured PME was treated with PNGase F and then subjected

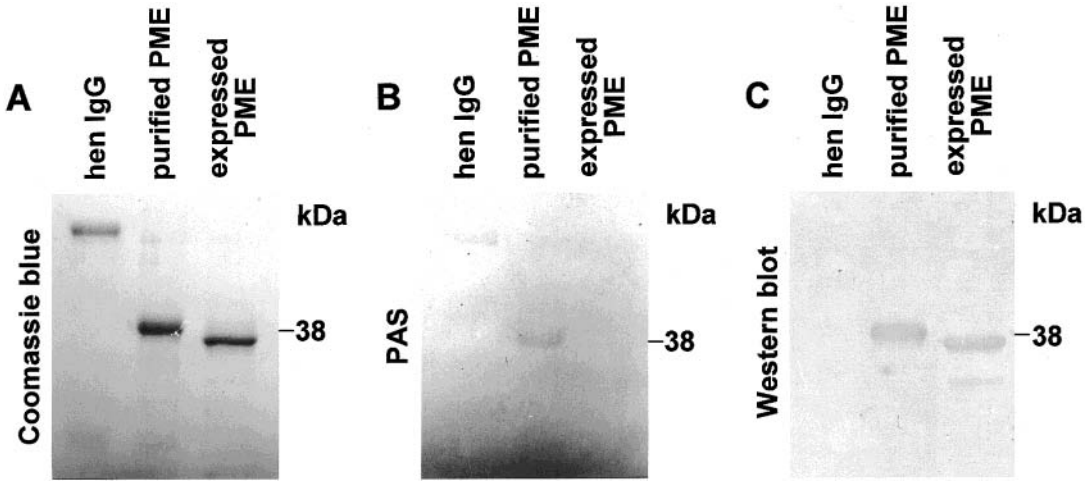

Figure 5. SDS–PAGE, Western blot and PAS carbohydrate staining of jelly fig PME. Hen egg IgG (a known glycoprotein), jelly fig PME purified from achenes, and overexpressed PME purified from *E. coli* cells (5 μg for each) were resolved in three replicated SDS–PAGE gels. The gel was subjected to Coomassie blue staining (A), immunodection using antibodies against PME purified from jelly fig achenes (B), and PAS carbohydrate staining (C).

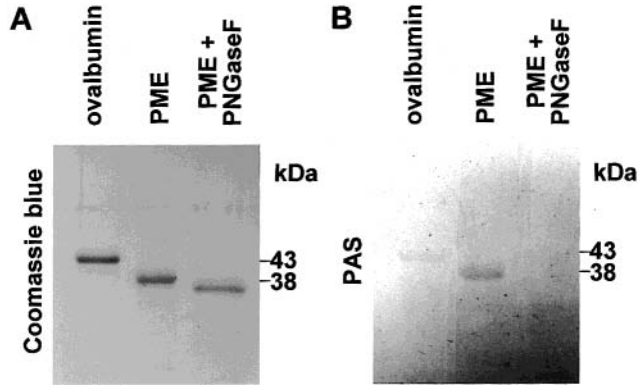

Figure 6. SDS–PAGE of jelly fig PME cleaved by PNGase F. Ovalbumin (a known glycoprotein) and purified jelly fig PME (5 μg for each) treated with or without PNGase F were resolved in two duplicated SDS–PAGE gels. The gel was subjected to Coomassie blue staining (A) or PAS carbohydrate staining (B).

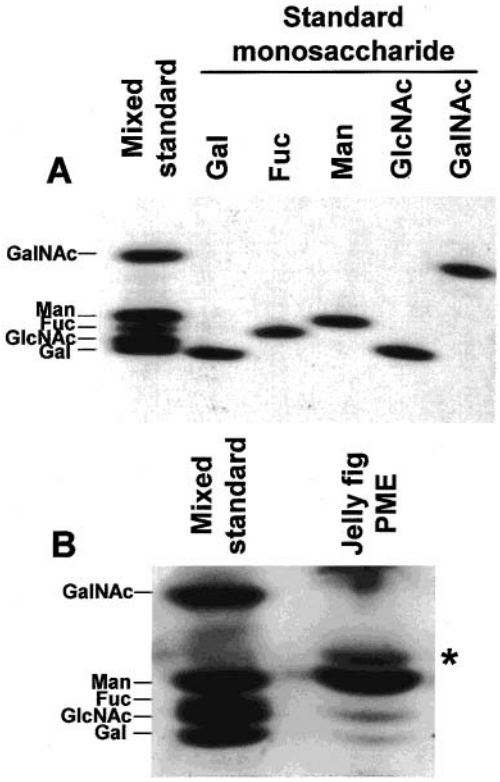

Figure 7. FACE gel analysis of monosaccharide composition of jelly fig PME glycan. (A) Mixed and individual fluorescence-labeled monosaccharide standards (D-galactose, L-fucose, D-mannose, *N*-acetylgalactosamine, and *N*-acetylglucosamine) were resolved in an FACE gel. (B) Neutral monosaccharides released from jelly fig PME glycan were compared with the mixed monosaccharide standards. The existence of α-D-methylmannoside due to the sample preparation (see Materials and Methods for details) is indicated by a star symbol.

to SDS−PAGE analysis to observe the change in molecular mass of PME before and after enzymatic digestion (**Figure 6A**). In an exhaustive digestion, the purified PME (38 kDa) was converted to a polypeptide close to 35kDa, equivalent to the size of overexpressed PME in *E. coli*. In contrast with intact jelly fig PME which was positively stained for the residence of carbohydrate, the remaining fragment (35 kDa) of jelly fig PME completely cleaved by PNGase F could no longer be stained by the PAS reaction (**Figure 6B**). This result suggests that jelly fig PME is an N-linked glycoprotein and possesses approximately 10% carbohydrate.

Monosaccharide Composition of the Polysaccharide Attached to Jelly Fig PME. To investigate monosaccharide composition of the attached carbohydrate, the polysaccharide of jelly fig PME was hydrolyzed to free monosaccharides, fluorescence-labeled, and then subjected to fluorophore-assisted carbohydrate electrophoresis. In comparison with standard monosaccharides, the released monosaccharides were estimated to comprise 72.75% mannose, 9.19% galactose, and 18.06% *N*-acetylglucosamine (**Figure 7**).

DISCUSSION

In this study, a fast and simple method was developed to purify PME from jelly fig achenes. PME was extracted from jelly curd instead of extracting it directly from achenes, in a manner similar to that for proteins eluted from polyacrylamide gels (*22*). Compared with the previous method developed by Lin et al. (*9*), this new method provides the same purity but a higher recovery yield in a much shorter period of time (3 days vs 5 h). Removal of pectin by forming jelly curd is a key step for the significant improvement of PME purification in the new method because the presence of pectin severely interferes with

the outcome of PME purification during ammonium sulfate precipitation and column chromatography.

In our preparation, a minor PME isoform of slightly lower molecular mass was consistently observed (**Figure 2**). Ratio of the major and the minor PMEs varies substantially in jelly fig achenes cultivated in different locations. This minor PME possesses enzymatic activity and can be recognized by antibodies against the major PME (data not shown). In both kiwi (*11*) and orange (*14*), PME isoforms of different glycosylation have been reported. Whether the minor PME was encoded by a different homologous gene or simply resulted from different glycosylation of the same gene product remains to be investigated.

According to this study, jelly fig PME is presumably N-glycosylated with approximately 10% carbohydrate (roughly equivalent to 3 kDa) in its mature protein. On the basis of FACE quantitative analysis, the ratio of mannose/*N*-acetylglucosamine/galactose is 8:2:1 (**Figure 7**). Taken together, the carbohydrate attached to jelly fig PME appears to consist of 22 hexoses, including 16 mannose, 4 *N*-acetylglucosamine, and 2 galactose residues, with a calculated molecular mass of 3,728 Da. The monosaccharide composition suggests that the carbohydrate moiety of jelly fig PME possibly forms a complex-type glycan structure, which contains unique galactose residues linked to the terminal *N*-acetylglucosamine units in almost all plant N-glycans (*23*). In some plant glycan structures, xylose is present as a minor constituent. Xylose, if present, comigrates with mannose and thus cannot be identified in the method of our monosaccharide analysis. Therefore, it is possible that xylose may be present as a minor constituent in the carbohydrate moiety of jelly fig PME. Currently, we are trying to determine the sequence of polysaccharide attached to jelly fig PME in a collaborative study. The monosaccharide composition will be recalculated when the attached carbohydrate is sequenced.

In plants, it has been shown that N-linked glycans significantly influence conformation, stability, and biological activity of glycoproteins (*24*). Presumably, glycosylation is essential for the folding and/or enzymatic activity of jelly fig PME, as its overexpressed polypeptide in *E. coli* is devoid of activity (*15*). For a functional expression of jelly fig PME, we are attempting to express this enzyme in yeast and plants. In our preliminary result, jelly fig PME expressed in yeast was hyper-glycosylated with slightly reduced enzymatic activity (data not shown).

In our first attempt to examine if jelly fig PME is an N-linked glycoprotein using PNGase F digestion, the purified (nondenatured) PME was not susceptible to the enzymatic cleavage. Subsequently, we found that jelly fig PME could be cleaved by PNGase F only after it was denatured by heating at 100 °C with 1% SDS and 5 mM β-mercaptoethanol. Possibly, the polysaccharide attached to jelly fig PME was hidden within the interior or tightly associated with the surface of the protein, and thus not accessible to PNGase F digestion.

ABBREVIATIONS USED

FACE, Fluorophore-assisted carbohydrate electrophoresis; PAS, periodic acid-Schiff; PME, pectin methylesterase; PVPP, polyvinylpolypyrrolidone.

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