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Determination of N-Glycosylation Site and Glycan Structures of Pectin Methylesterase in Jelly Fig (*Ficus awkeotsang*) Achenes

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Pectin methylesterase (PME) in jelly fig (*Ficus awkeotsang*) achenes is an N-glycosylated enzyme responsible for the gelation of jelly curd. A recombinant jelly fig PME was overexpressed in *Escherichia coli* and confirmed by immunodetection and LC−nanoESI−MS/MS analysis. To identify the N-glycosylation site, native PME and its deglycosylated and recombinant forms, which lacked glycan, were purified and subjected to comparative MALDI-MS mapping of the corresponding tryptic fragments. The results showed that N-glycosylation occurred at Asn_{153} of the mature jelly fig PME, the only glycosylation site predicted by its sequence analysis. The major N-glycans released from the native PME by PNGase F were identified by MS/MS analyses as xylosylated, noncore fucosylated pauci-mannose, and complex-type structures. Molecular modeling of the 3D structure of jelly fig PME indicated that the N-glycan was putatively attached to the back region of the active site of this enzyme.

KEYWORDS: Glycan structure; jelly fig; mass analyses; N-glycosylation; pectin methylesterase

INTRODUCTION

Pectin is a major hydrocolloid used in the manufacture of various products including food, wine, juice, cosmetics, and medicine. Pectin methylesterase (PME) (EC 3.1.1.11), a ubiquitous enzyme in plants, de-esterifies the methoxylated pectin in the plant cell wall and is responsible for pectin degradation with the combined activities of polygalacturonase and pectate lyase (*1*). It is assumed that PME plays an important role in plant cell growth and differentiation (*2*) and may also serve as a host cell receptor involved in cell-to-cell movement of invading viruses (*3*,*4*). Owing to its enzymatic activity on pectin, PME has been used in the food, juice, and wine industries (*5*,*6*).

An acidic PME isolated from jelly fig (*Ficus awkeotsang* Makino) achenes has been characterized as a thermostable (up to 65 °C) enzyme responsible for the gelation of water extract from jelly fig achenes (*7*). The gelled product, jelly curd, is utilized for preparation of a popular drink in Taiwan. 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 (*8*). Jelly fig PME retains its enzymatic activity over a broad pH range, particularly in acidic environments where most known PME enzymes from various plant species are inactivated (*9*). Moreover, its enzymatic activity can be fully preserved after a long period (e.g., six months) of storage at 4 °C. These biochemical and biophysical characteristics of jelly fig PME suggest its potential applications in industries.

In plants, it has been shown that N-linked glycans significantly influence conformation, stability, and biological activity of glycoproteins (*10*). PME isoforms with different glycosylation have been reported in some plant species, such as kiwi and orange, and the difference in glycosylation seems to affect their thermostability and pectin affinity (*11*−*13*). The native PME isolated from jelly fig achenes has been determined as an N-linked glycoprotein (*14*). While the recombinant PME expressed in *Escherichia coli* lacked glycosylation and enzymatic activity, the recombinant PME expressed in *Pichia pastoris* was hyperglycosylated and functionally active (*15*). Probably, glycosylation is essential for the folding or thermostability of jelly fig PME.

In this study, we aimed to identify the N-glycosylation site in jelly fig PME and to determine the chemical structures of glycans attached to this enzyme by mass spectrometry. For a better understanding of this N-glycosylation, the 3D structure of jelly fig PME was simulated by homology modeling to reveal the relative locations of the attached glycan and the enzymatic active site.

MATERIALS AND METHODS

Purification of PME from Jelly Fig Achenes. Jelly fig (*Ficus awkeotsang* Makino) achenes were purchased from local growers. Jelly curd was prepared by a hand-washing method, and jelly fig PME was purified from jelly curd according to the methods developed by Ding et al. (*14*) with minor modification. After extraction of PME from jelly curd, the insoluble pectin polymers were removed by centrifugation. Extracted PME in the soluble supernatant was precipitated with ammonium sulfate

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at a final concentration of 40% 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 the minimal buffer and then dialyzed against 1 L of the minimal buffer supplemented with 0.15 M NaCl at 4 °C for 30 min. After dialysis, the PME was further purified by a DEAE−Sephadex A-25 column (GE Healthcare). The eluent was concentrated by an Amicon concentrator (Amicon) under nitrogen gas.

Overexpression of Recombinant Jelly Fig PME. The cDNA clone of jelly fig PME as described previously (*9*) was constructed in the fusion expression vector, pET29a(+) (Novagen), using an *Nde*I site at the initial methionine position and a *Hin*dIII site in the polylinker of the vector. The recombinant fusion protein comprised the mature jelly fig PME fused with a C-terminal appendix of 12 amino acid residues (LAAALEHHHHHH). The recombinant plasmid was transformed into *E. coli* strain BL21(DE3). Overexpression was induced by adding with 1 mM isopropyl *β*-D-thiogalactoside (IPTG) in a bacteriophage T7 RNA polymerase/promoter system. The *E. coli* cells were harvested 3 h after induction and then crushed by sonication in 10 mM sodium phosphate buffer, pH 7.5. After centrifugation, the pellet containing the recombinant PME was dissolved in a buffer containing 6 M guanidine-HCl and purified by immobilized metal affinity chromatography (BD Biosciences, TALON metal affinity resins) according to the user's manual. The purified recombinant PME was concentrated by the centrifugal filter units (Millipore, Amicon Ultra-15).

Antibody Preparation and Western Blotting. Recombinant jelly fig PME resolved in SDS−PAGE using 12.5% polyacrylamide was eluted according to a modified gel extraction procedure (*16*). Polyclonal antibodies against the recombinant PME were raised in chickens, and immunoglobulins were purified from egg yolks for the immunoassay (*17*). In the immunoassay, proteins resolved in the SDS−PAGE gel were transferred onto a nitrocellulose membrane (Pall, BioTrace NT) in a Bio-Rad Trans-Blot system according to the manufacturer's instructions. The membrane was subjected to immunodetection using secondary antibodies conjugated with alkaline phosphatase (Abcam, goat polyclonal to chicken IgY) and then incubated with the BCIP/NBT color development solution (PerkinElmer).

PAS Staining for Glycosylation. Purified jelly fig PME was denatured in a solution of 0.5% SDS and 40 mM dithiothreitol (DTT) for 10 min at 100 °C. To examine whether the attached glycans are N-glycosylated to the protein, 10 *μ*g of the above denatured PME was treated with 500 U of PNGase F (New England BioLabs) at 37 °C overnight in a buffer containing 1% NP-40 and 50 mM sodium phosphate, pH 7.5. The PME before and after PNGase F digestion was resolved by SDS−PAGE and subjected to staining of carbohydrate using an improved periodic acid−Schiff (PAS) reaction (*18*).

In-Gel Digestion of PME. Native jelly fig PME, as well as its deglycosylated and recombinant forms, was manually excised from the gel, and the gel slice was ground into pieces. The pieces containing PME samples were reduced with 10 mM DTT for 45 min at 56 °C and alkylated with 55 mM iodoacetamide for 30 min in the dark at room temperature (24 °C). Following dehydration in acetonitrile, the protein bands were dried and digested with 0.1 *μ*g of TPCK-treated modified porcine trypsin (Promega) overnight at 37 °C. The supernatant containing tryptic peptides was combined with two more extracts of the gel by 50% acetonitrile/1% trifluoroacetic acid for the following MS analysis.

Permethylation of Glycans from Jelly Fig PME. Native jelly fig PME was digested with trypsin and then treated with PNGase F as described in the previous in-gel digestion and glycosylation analysis. The two fractions of glycans and deglycosylated peptides desalted and separated by using a C18 cartridge (Waters, Sep-pak SPE) were eluted with 5 mL of 0.1% formic acid and 5 mL of 75% acetonitrile/1% formic acid, respectively. The de-N-glycosylated peptides after freeze-drying were subjected to MALDI-MS analysis for verifying the implicated N-glycosylation site. The glycans left in a glass vial after freeze-drying were permethylated by the dimethyl sulfoxide method (*19*). The permethyl derivatives extracted in chloroform and repeatedly washed with water were subjected to MS analysis for the glycan profiling and sequencing.

MS and MS/MS Analysis. Tryptic peptides derived from in-gel digestion of the native or recombinant PME were analyzed by LC−nanoESI−MS/MS on a Q-TOF Ultima API (Micromass, Manchester, U.K.) instrument for protein identification. Raw data files were processed by ProteinLynx 2.2 and converted into pkl files for searching against the Swiss-Prot protein sequence database using Mascot software (Matrix Science Ltd., London, U.K.). MALDI-TOF MS profiling of permethylated N-glycans was performed on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) using the 2,5-dihydroxybenzoic acid matrix (10 mg/mL in water, mixed 1:1 with sample dissolved in acetonitrile), spotted on the target plate, air-dried, and recrystallized on the plate with acetonitrile. High-energy MALDI-CID MS/MS sequencing of the permethylated glycans was performed on the same ABI 4700 Proteomics Analyzer exactly as described previously (*20*). The N-glycosylation site of PME was verified by a MALDI micro MX mass spectrometry (Waters, Milford, MA) using the sinapinic acid matrix (10 mg/mL in water, mixed 1:1 with sample dissolved in acetonitrile). The implicated N-glycosylation site was manually interpreted against the known molecular weight of putative deglycosylated peptide with a criterion of asparagine to aspartate conversion.

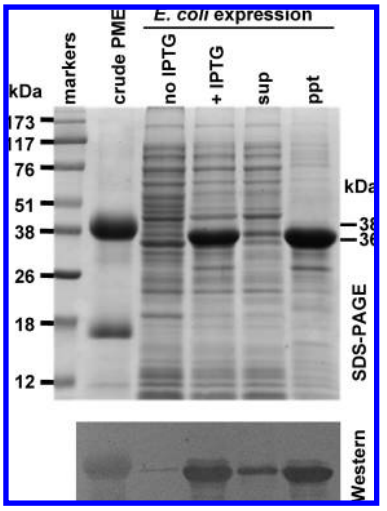

Figure 1. SDS−PAGE and Western blot of the recombinant PME in *E. coli*. Along with crude PME isolated from jelly fig achenes, soluble (sup) and insoluble (ppt) proteins extracted from *E. coli* cells with or without IPTG induction for overexpression of the recombinant PME were analyzed by SDS−PAGE. The molecular masses of marker proteins are indicated on the left, and those of native and recombinant PME on the right. A duplicate gel was transferred onto nitrocellulose membrane and subjected to immunodetection using antibodies against the recombinant PME.

Table 1. Fragments of Native and Recombinant PME Identified by LC−nanoESI−MS/MS Analysis

	residues	sequence
native PME	6−14	DIEPDIVVAK
	16−36	DGSGDYETLNEAVAAIPDNSK
	38−44	RVIVLVR
	45−58	TGIYEENVDFGYQK
	60−77	NVMLVGEGMDYTIITGSR
	114−120	YQAVALR
	121−130	IGADETVINR
	133−146	IDAYQDTLYPHNYR
	182−192	GQENTITAQGR
	223−230	SYLGRPWK
	262−269	TLFYGEYR
recombinant PME	16−36	DGSGDYETLNEAVAAIPDNSK
	45−58	TGIYEENVDFGYQK
	60−77	NVMLVGEGMDYTIITGSR
	114−120	YQAVALR
	121−130	IGADETVINR
	182−192	GQENTITAQGR
	262−269	TLFYGEYR

Sequence Alignment and Molecular Modeling. Sequence comparison of PMEs from jelly fig, carrot, and kiwi was performed with the GenBank sequences using the ClustalW method as part of Megalign (DNASTAR Inc., Madison, WI). Comparative modeling was performed by the Build Homology Models program and minimized by the Loop Refinement program in the Discovery Studio 2.1 software (Accelrys, San Diego, CA). The models of jelly fig and kiwi PMEs were built by using the carrot PME (Protein Data Bank [PDB] code 1GQ8) as a structure template.

RESULTS

Production of Recombinant Jelly Fig PME in *E. coli*. A cDNA fragment encoding the mature protein of jelly fig PME was constructed in a fusion vector and overexpressed in *E. coli* to generate a recombinant polypeptide with a C-terminal His-tag. As revealed in SDS−PAGE, the recombinant PME, confirmed by immunoassay, was predominantly present in the pellet of *E. coli* cell lysate (**Figure 1**). The identification of native jelly fig PME and the recombinant one was also confirmed by LC−nanoESI−MS/MS analysis (**Table 1**). Despite fusion with a C-terminal His-tag of 12 amino acid residues, the recombinant PME (36 kDa) was smaller than the native N-glycosylated PME (38 kDa) isolated from jelly fig achenes.

Purification of Native, Deglycosylated, and Recombinant PME. To identify the N-glycosylation site in jelly fig PME by mass spectrometric analysis, native PME and the recombinant one were purified by DEAE column chromatography and immobilized metal affinity chromatography, respectively. Deglycosylated PME was obtained by treating the native PME purified from jelly fig achenes with PNGase F. In contrast with native PME, the deglycosylated or the recombinant PME that lacked glycan could not be stained in the improved PAS reaction, though all three PME polypeptides were detected by immunoassay (**Figure 2**). The deglycosylated PME and the recombinant one were comparable in size, while the native N-glycosylated PME was slightly larger.

Identification of the N-Glycosylation Site in Jelly Fig PME. Sequence analysis indicates that a putative N-glycosylation site at Asn_{153} of jelly fig PME is the only glycosylation site on this enzyme (**Figure 3**). Apparently, this N-glycosylation site in jelly fig PME is not universally present in plant PMEs because neither carrot PME nor kiwi PME possesses this site. Similar mass spectrometric patterns were observed for the tryptic fragments of native, deglycosylated, and recombinant PMEs (**Figure 4A**). As expected, a tryptic peptide (Asp_{151}−Arg_{177}) containing the N-glycosylation site (Asn_{153}) was observed in both deglycosylated PME (*m*/*z* 3059.3) and recombinant PME (*m*/*z* 3058.2) but not in native PME, which possessed two extra tryptic fragments of higher molecular masses (*m*/*z* 4083.6 and 4286.3) possibly due to the attachment of glycans to the N-glycosylation site (**Figure 4B**). The 1 Da difference between the mass of Asp_{151}−Arg_{177} in deglycosylated PME (*m*/*z* 3059.3) and that in recombinant PME (*m*/*z* 3058.2) was in agreement with the conversion of asparagine to aspartate at the N-glycosylation site cleaved by PNGase F.

Chemical Structures of the Glycans Released from Jelly Fig PME. N-Glycans released from native PME by PNGase F cleavage were permethylated for MALDI-MS mapping (**Figure 5**). Five major signals were observed, which could be tentatively assigned as sodiated molecular ions of typical plant N-glycans (*10*) based on molecular compositions inferred from

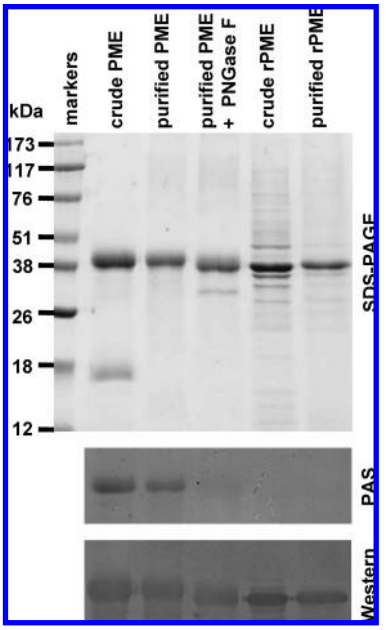

Figure 2. SDS−PAGE, PAS carbohydrate staining, and Western blot of native, deglycosylated, and recombinant PMEs. Crude and purified forms of native, deglycosylated, or recombinant PME (rPME) were resolved in three replicated SDS−PAGE gels. The gel was subjected to Coomassie blue staining, PAS carbohydrate staining, and immunodection using antibodies against rPME. The molecular masses of marker proteins are indicated on the left.

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Jelly fig  QTLGKDIEPDIVVAKDGSGDYETLNEAVAAIPDNSKKRVIVLVRTGIYEENVDFGYQKKNVMLVGEGMDYTIITGSRNVVDGSTT  85
Carrot     --QSSTVTPNVVVAADGSGDYKTVSEAVAAAPEDSKTRYVIRIKAGVYRENVDVPKKKKNIMFLGDGRTSTIITASKNVQDGSTT  83
Kiwi       -TAVTDIVPDVVVAKDGSGNFTTVGAAVAAAKDSSTARFVIYIKEGAYFEYVDVDKKKTNLMFIGDGIGKTWIKGNRSVVDGWTT  84

                                                                                    PME motif
Jelly fig  FDSATVAAVGDGFIAQDICFQNTAGPEKYQAVALRIGADETVINRCRIDAYQDTLYPHNYRQFYRDCNITGTVDFIFGNAAVVFQ 170
Carrot     FNSATVAAVGAGFLARDITFQNTAGAAKHQAVALRVGSDLSAFYRCDILAYQDSLYVHSNRQFFINCFIAGTVDFIFGNAAVVLQ 168
Kiwi       FRSSTVAVVGTGFIARGISFENYAGPSKHQAVALRSGADFSAFYQCSFVGYQDTLYVHSLRQFYSECDVYGTIDFIFGNAAAVLQ 169

Jelly fig  NCNLIPRKQMKGQENTITAQGRTDPNQNTGTSIQNCEIFASADLEPVEDTFKSYLGRPWKEYSRTVVMESYISDVIDPAGWLEWD 255
Carrot     DCDIHARRPGSGQKNMVTAQGRTDPNQNTGIVIQKSRIGATSDLQPVQSSFPTYLGRPWKEYSRTVVMQSSITNVINPAGWFPWD 253
Kiwi       KCNLYARKPNENQKNIFTAQGRDDPNQNTGISILNCKVAAAADLIPVLSSFKTYLGRPWKEYSRTVFLLSQMESLIDPAGWLEWS 254

Jelly fig  RDFALKTLFYGEYRNGGPGSGTSERVKWPGYHVITSPEVAEQFTVAELIQGGSWLGSTGVDYTAGLYA                  323
Carrot     GNFALDTLYYGEYQNTGAGAATSGRVTWKGFKVITSSTEAQGFTPGSFIAGGSWLKATTFPFSLGL                   319
Kiwi       GDFALTTLYYREYKNTGPGSNTTARVTWPGYAVTTNETEVIQFTVGNFIQGSQWLTSYNIPVYLNLT                  321
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Figure 3. Sequence alignment of PMEs from jelly fig, carrot, and kiwi. The amino acid number for the last residue in each line is listed for each species. Residues involved in the active site of PME were highlighted. Putative N-glycosylation sites were shaded in gray. The conserved PME motif is indicated on top of the sequences. The accession numbers of the aligned sequences are jelly fig, P83947; carrot, P83218; kiwi, P85076.

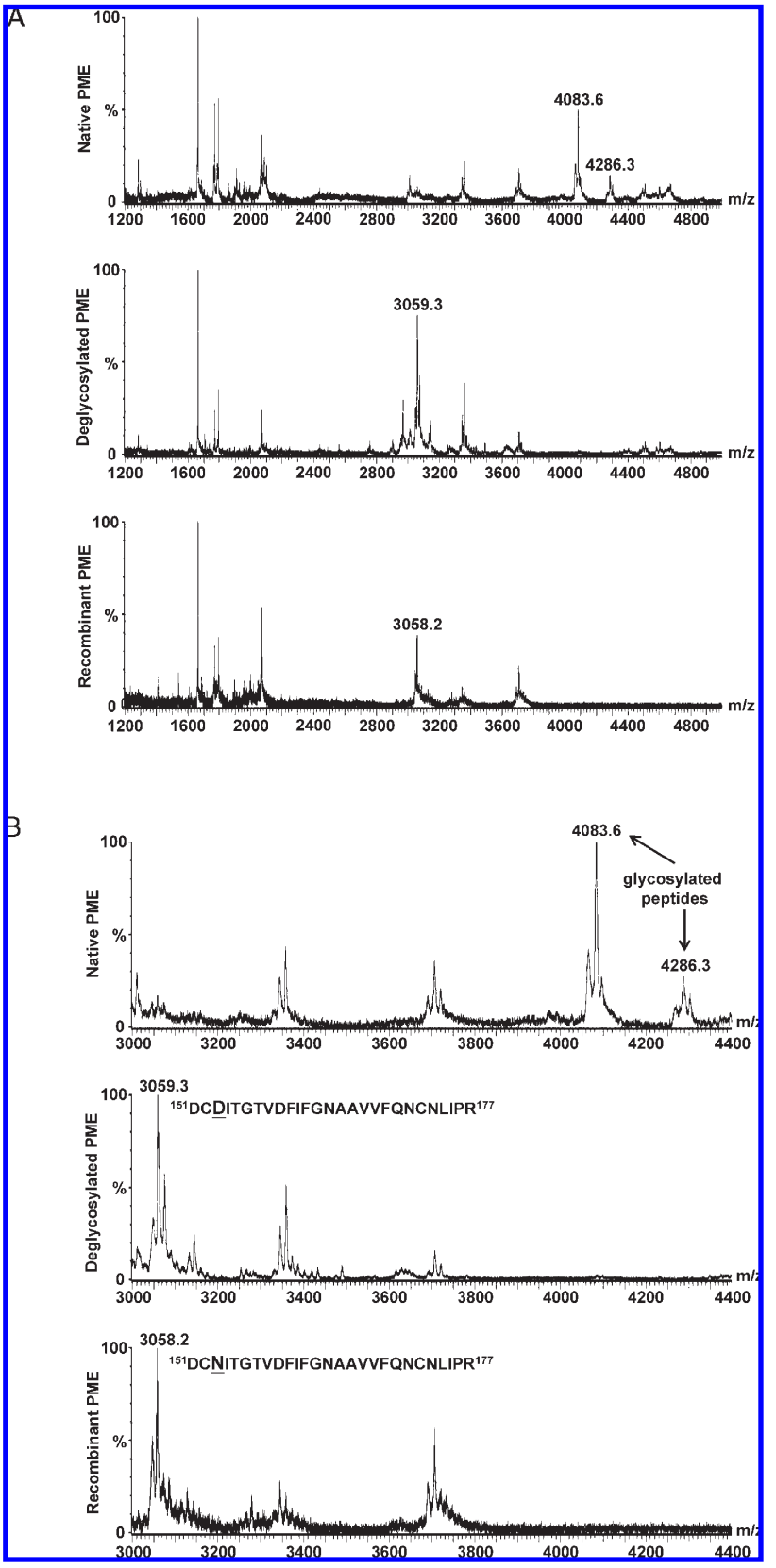

Figure 4. MALDI-TOF MS spectra for identifying the N-glycosylation site in jelly fig PME. Tryptic peptides of native, deglycosylated, and recombinant PMEs were analyzed by MALDI-TOF MS. The spectra were displayed with the molecular masses ranging from 1200 to 5000 Da (**A**) or in enlarged panels from 3000 to 4400 Da (**B**). Distinct peaks found between glycosylated (native) and unglycosylated (deglycosylated or recombinant) PMEs were indicated by their *m*/*z* values.

their *m*/*z* values. The structures of the three more abundant components, corresponding to xylosylated, noncore fucosylated pauci-mannose, and complex-type structures (*m*/*z* 1331.8, 1577.0, and 1822.1), were further verified by high-energy CID MS/MS analysis on a MALDI TOF-TOF instrument (**Figure 6**). In each case, the overall sequences were unambiguously defined by

complementary series of reducing (^{1,5}X and Y ions) and nonreducing terminal ions (B and C or C″ ions), as schematically illustrated, which also localized the Xyl onto the β-Man of the trimannosyl core. In the case of complex type structures (**Figure 6B**,**C**), the presence of nonreducing terminal HexNAc is evident from the prominent Y ions, as well as the B_1 ion corresponding to HexNAc at *m*/*z* 282. The other two minor components (*m*/*z* 1117.7, 1416.9) not subjected to further MS/MS analysis are most likely the nonxylosylated counterparts of the established structures.

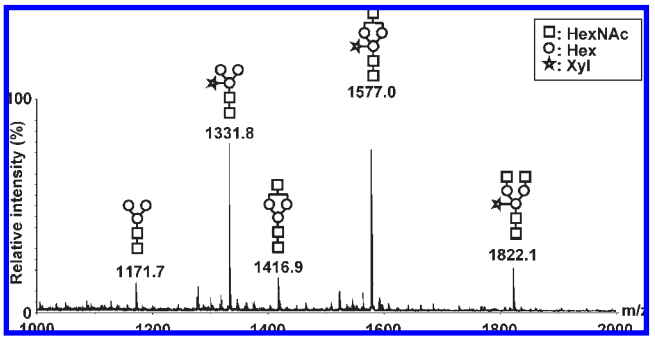

Figure 5. MALDI MS profile of permethylated N-glycans released from jelly fig PME. N-glycans of jelly fig PME purified by a C18 cartridge after PNGase F treatment were subjected to permethylation. All labeled molecular ion signals belong to $[M + Na]^+$ forms. Symbols of the different carbohydrates were □, HexNAc (GlcNAc); ○, Hex (Man); and ★, Xyl.

Homology Modeling of the 3D structure of Jelly Fig PME. The 3D structure of jelly fig PME, as well as that of kiwi PME, simulated by homology modeling using the crystal structure of the carrot PME as a template belongs to the family of right-handed parallel β-helical proteins (**Figure 7**). Conservative active site for enzymatic activity is present in all three PME structures. An N-linked glycan is attached to the unique glycosylation site of jelly fig PME in the back region of its active site. In contrast, no glycosylation occurs in the carrot PME, while four N-linked glycans are attached to both ends of the kiwi PME.

DISCUSSION

According to the unambiguous analysis in this study, the N-glycans of jelly fig PME belong to the pauci-mannose and

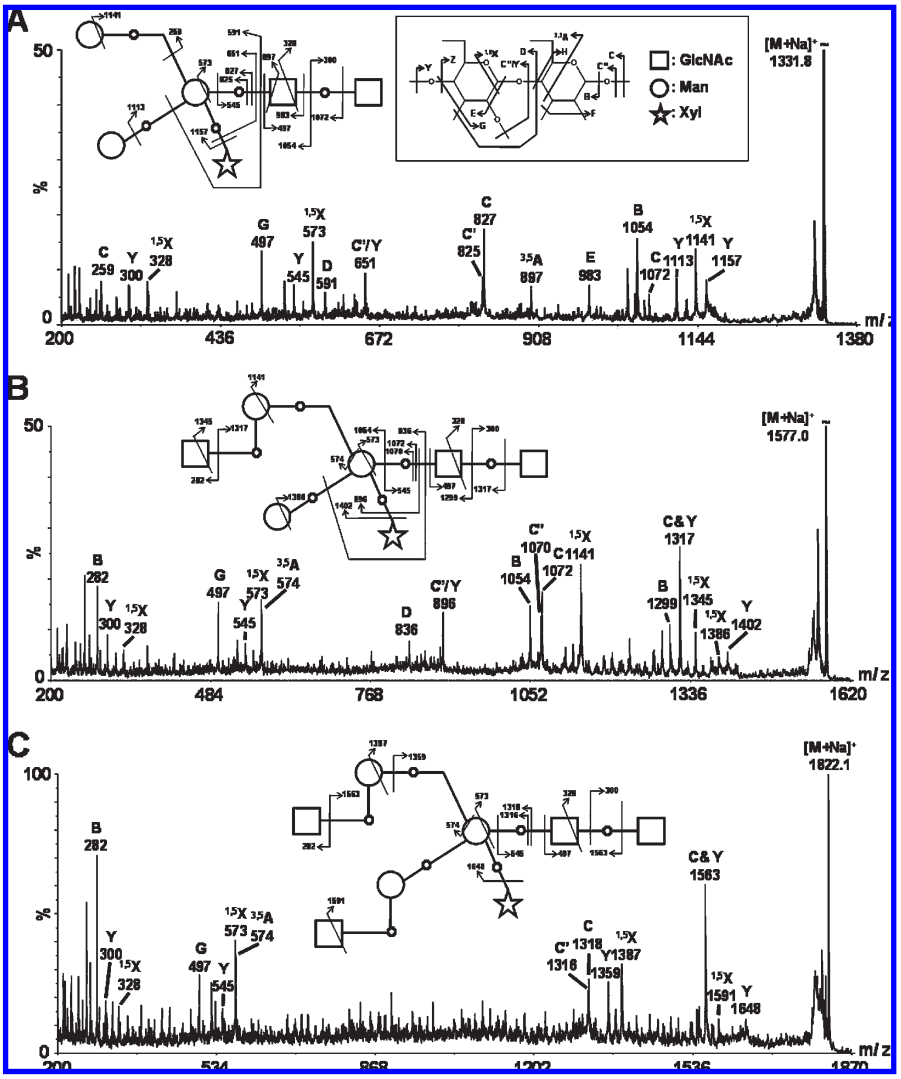

Figure 6. MALDI TOF-TOF MS/MS sequencing of the N-glycans from jelly fig PME. The three major N-glycan peaks (*m*/*z* 1331.8, 1577.0, and 1822.1) detected by the MALDI MS profiling (**Figure 5**) were subjected to MS/MS analysis. The schematic drawings illustrate how the various glycosyl residues and linkage-specific cleavages collectively contributed to unambiguous structural assignment. For the structure with single terminal HexNAc (*m*/*z* 1577.0), the prominent ^{3,5}A ion at *m*/*z* 574 and the D ion at *m*/*z* 836 formed at the β-Man collectively indicated that the terminal HexNAc was primarily carried on the six-arm although the data do not rule out the presence of the alternative isomer. Symbols of the different carbohydrates were □, GlcNAc; ○, Man; and ☆, Xyl.

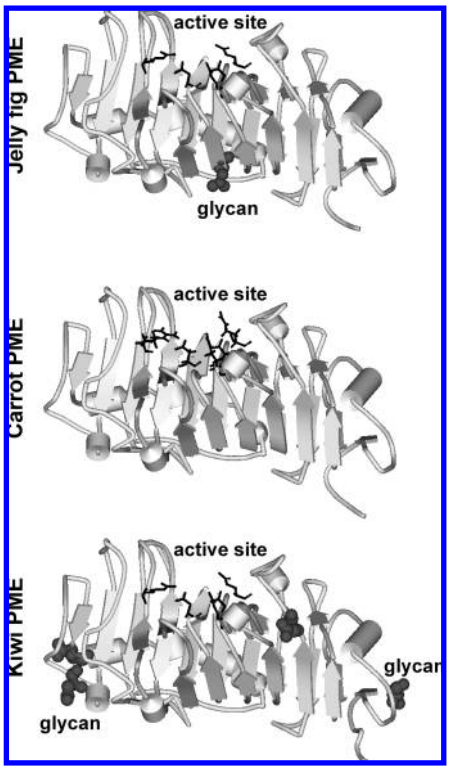

Figure 7. Three-dimensional structures of jelly fig, carrot, and kiwi PMEs. The structural models of jelly fig PME and kiwi PME were predicted by using carrot PME (PDB code 1GQ8) as a structural template. In the 3D structures, amino acids of the active site and those of the N-glycosylation site are displayed as sticks and balls, respectively.

complex-type structures without fucosylation on the proximal GlcNAc of the core $Man_3GlcNAc_2$. This result was in accord with the observation in the fluorophore-assisted carbohydrate analysis as described by Ding et al. (*14*). It also explained why the N-glycans in jelly fig PME could be removed from the protein not only by PNGase A (data not shown) but also PNGase F (**Figure 2**) since they do not contain core α1,3-fucosylation often found in plant glycoproteins that would render them resistant to PNGase F digestion.

Similar to most PMEs from higher plants, the mature protein of jelly fig PME is first synthesized as a precursor protein in the form of pre-pro-protein (*21*). The presequence contains a cleavable signal sequence for targeting to endoplasmic reticulum (ER), and the pro-sequence contains a PME inhibitor domain. The pro-sequence and the mature PME are linked by a processing motif (RRLL or RKLL) that is putatively cleaved by subtilisin-like proteases. Following this processing model, we expressed the recombinant PME in a mature form as shown in **Figure 3**, though an 11-residue fragment, IEPDIVVAKDG (residues of 7−17 in the mature jelly fig PME) was obtained by direct N-terminal sequencing of native jelly fig PME (*9*).

Sequence analysis indicates that the unique N-glycosylation site (Asn_{153}) of jelly fig PME is located right next to the conserved PME motif (residues 154−163) comprising the active site of the enzyme (**Figure 3**). However, 3D homology modeling indicates that this unique N-glycosylation site of jelly fig PME is located in the back region of its enzymatic active site (**Figure 7**). Therefore, it is unlikely that the N-glycans attached to jelly fig PME are involved in the direct interaction and stabilization with the pectin substrate for enzymatic activity. Being not present in many other plant PMEs, the N-glycosylation site of jelly fig PME is apparently not indispensable for its protein folding of the right-handed parallel β-helical domain. Whether the unique N-glycosylation site of jelly fig PME is associated with its distinct thermostability (up to 65 °C) remains to be studied.

In our previous studies, the recombinant PME expressed in *P. pastoris* was hyperglycosylated and functionally active (*15*), whereas PME lacked glycosylation and enzymatic activity when expressed in *E. coli* (*9*). According to the N-glycosylation synthesis (*22*), the hyperglycosylation in *P. pastoris* presumably occurs at the unique N-glycosylation site of jelly fig PME and belongs to the high-mannose-type N-glycan. In this study, five different N-glycans were observed in native jelly fig PME, though their relative enzymatic activity and thermostability have not been quantitatively evaluated. It appears that the activity of jelly fig PME is not much affected by the size and shape of N-glycans attached to it, which seems to be in agreement with the observation that the N-glycosylation site is located in the back region of the active site of this enzyme.

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