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Functional Expression in *Pichia pastoris* of an Acidic Pectin Methylesterase from Jelly Fig (*Ficus awkeotsang*)

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A cDNA fragment encoding an acidic pectin methylesterase (PME) of jelly fig achene was successfully expressed in *Pichia pastoris* under the control of the glyceraldehydes-3-phosphate dehydrogenase promoter. The recombinant PME was produced as a secretory protein by N-terminal fusion of a cleavable prepropeptide for signal trafficking, and thus easily harvested from the culture medium. Compared with native N-glycosylated PME (38 kDa) purified from jelly fig achenes, this recombinant PME (45 kDa) appeared to be hyperglycosylated. Activity staining indicated that the recombinant PME was functionally active. Yet the hyperglycosylated recombinant PME possessed thermostability and enzymatic capability over a broad pH range equivalent to those of the native PME. The success of functional production of this acidic jelly fig PME in *P. pastoris* has significantly broadened its applications in industry.

KEYWORDS: Functional expression; glycosylation; jelly fig achene; pectin methylesterase; *Pichia pastoris*

INTRODUCTION

Pectin is one of the most abundant polysaccharides on earth and 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, deesterifies the methoxylated pectin in the plant cell wall and is responsible for pectin degradation with the combined activities of polygalacturonase and pectate lyase (*1*). Owing to its enzymatic activity on pectin, PME is regarded as a critical enzyme in the food, cosmetic, and pharmaceutical industries (*2*, *3*).

An acidic PME isolated from jelly fig (*Ficus awkeotasang* Makino) achenes has been characterized as a thermostable (up to 65 °C) N-linked glycoprotein with 10% carbohydrate (*4*). It can retain its enzymatic activity over a broad pH range, particularly in acidic environments where most known PME enzymes from various plant species are inactivated (*5*, *6*). In addition, its 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 industry.

Purification of jelly fig PME from achenes is economically not as effective as the production of PME from fungi (*7*). Applications of jelly fig PME in industry would become feasible if this enzyme could be massively produced by well-established expression systems via genetic engineering. Previously, expression of jelly fig PME in *Escherichia coli* has resulted in the formation of inclusion bodies (*6*). The insoluble PME (35 kDa) overexpressed in *E. coli* was smaller than the native jelly fig PME (38 kDa) and exhibited no detectable enzymatic activity, implying that *E. coli* expression systems are not suitable for this N-glycosylated protein. We thus explored the possibility of using yeast *Pichia pastoris*, a system proved effective for expressing recombinant proteins with proper glycosylation modification and a high expression level in soluble form (*8*, *9*), to produce the properly folded and functional PME.

The recombinant jelly fig PME successfully expressed and secreted into the cultured medium of *P. pastoris* X-33 transformants seemed to be hyperglycosylated, in comparison with native PME, yet possessed equivalent enzymatic capability and thermostability. Jelly fig PME expressed in *P. pastoris* appears to be suitable for commercial utilization.

MATERIALS AND METHODS

Plasmid Construction and Transformation. The cDNA clone of jelly fig PME was constructed in the plasmid pGAPZαC, a *P. pastoris* vector used for expressing a target protein as a secretory polypeptide by N-terminal fusion with the cleavable *Saccharomyces cerevisiae* α-factor prepropeptide (*10*). The resulting plasmid, pGAPZα-PME, was linearized with *Bgl*II for easy homologous recombination between the vector and the region of homology within the genome. The vector was mixed with 80 μL of yeast cells washed with sterile water and aqueous sorbitol solution, and then transformed into *P. pastoris* X-33 cells by electroporation. The cells were pulsed using a 0.2 cm cuvette and a GenePulser (Bio-Rad). The charging voltage, capacitance, and resistance

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were 1500 V, 25 μF, and 200 Ω, respectively. pGAPZαC vector without the PME gene was also linearized and transformed into *P. pastoris* X-33 cells for negative control. The colonies were selected by plating on Zeocin-YPDS agar plates (20 g/L tryptone, 10 g/L yeast extract, 20 g/L dextrose, 182.2 g/L sorbitol, 20 g/L agar, and 100 μg/mL Zeocin). Plasmid construction and expression of jelly fig PME in *E. coli* were described previously (*6*).

Overexpression of Recombinant PME in *P. pastoris*. Using pGAPZα-PME, the expression of PME is regulated by the glyceraldehydes-3-phosphate dehydrogenase promoter. The chosen *P. pastoris* strains were cultivated with 5 mL of YPDS broth (20 g/L tryptone, 10 g/L yeast extract, 20 g/L dextrose, and 182.2 g/L sorbitol) containing 100 μg/mL Zeocin in a 50 mL flask using a shaking incubator (300 rpm) overnight at 30 °C, and then 1 mL of the resulting culture was inoculated into 50 mL of fresh YPD broth (20 g/L tryptone, 10 g/L yeast extract, and 20 g/L dextrose) in a 250 mL flask. It was then cultivated at 30 °C in a shaking incubator (300 rpm) for 4−5 days. The *P. pastoris* cells were excluded by 10 min of centrifugation at 3000*g*. The supernatant was collected and subjected to further purification.

Purification of Recombinant PME. The expressed PME in the soluble supernatant was precipitated with ammonium sulfate at a final concentration of 40−60% saturation and collected by centrifugation at 15000*g* for 30 min after the mixture was stirred at room temperature for 10 min. The precipitate was suspended in 10 mL of the minimal buffer containing 10 mM Tris−HCl, pH 7.5, and then dialyzed against 1 L of the minimal buffer supplemented with 0.15 M NaCl at 4 °C for 30 min.

Purification of PME from Jelly Fig Achenes. Jelly fig (*F. awkeotasang* 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. (*4*). 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 at a final concentration of 85% saturation and collected by centrifugation at 15000*g* for 30 min after the mixture was stirred 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 to near homogeneity by a DEAE-Sephadex A-25 column (Pharmacia). The eluent was concentrated by an Amicon concentrator to 1.5 mL, and then subjected to enzymatic activity assay.

Analysis of Proteins by SDS−PAGE and Western Blotting. Protein samples were resolved by SDS−PAGE (*11*) using 12.5% acrylamide. 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. Polyclonal antibodies against jelly fig PME were raised in chicken previously (*6*). In the immunoassays, proteins resolved in the SDS−PAGE gel were transferred onto the 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 (*12*).

Activity Staining of PME. The activity staining of PME was analyzed in either 1% agarose plate or 12.5% nondenaturing acrylamide gel electrophoresis containing 0.1% apple pectin. In the agarose plate for activity ring staining, the enzymes were allowed to diffuse at 30 °C for 1 h. Subsequently, the plate was washed with 0.1 M phosphate buffer, pH 7.5, and then stained with 0.02% ruthenium red in the minimal buffer. For gel staining, a nondenaturing acrylamide gel electrophoresis was performed at 4 °C for 5 h at a constant voltage of 50 V. After electrophoresis, the gel was immersed in 0.1 M phosphate buffer, pH 7.5, prior to activity staining with 0.02% ruthenium red in the minimal buffer.

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 (*13*). The assay comprised 0.2% apple pectin in 3 mL of reaction mixture, and activity was expressed as μmoles of acid produced per minute in 1 mL of incubation reaction mixture. To evaluate the relative activity of PME at various pH and temperature environments, equal PME amounts were applied to each buffer solution and assayed in the same conditions.

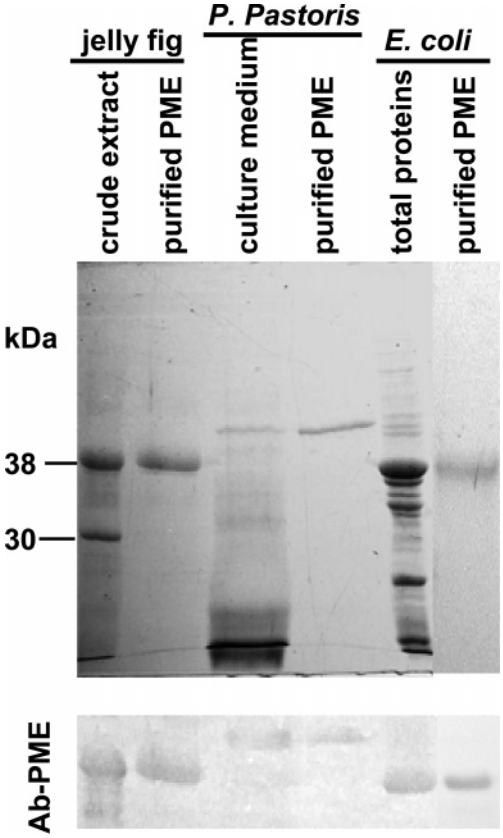

Figure 1. SDS−PAGE and Western blot analyses of native PME from jelly fig achenes and the recombinant PMEs expressed in *P. pastoris* and *E. coli*.

RESULTS

Expression of PME in *P. pastoris* as a Secretory Protein. As no enzymatic activity was detected in recombinant jelly fig PME expressed in *E. coli*, jelly fig PME was designed to be expressed in *P. pastoris*. The highest activity (~175.4 U/mg) of PME, expressed as a secretory protein in yeast by N-terminal fusion of a cleavable prepropeptide for signal trafficking, was observed 2 days after shaking cultivation. The recombinant PMEs expressed in *E. coli* and *P. pastori*, along with native PME purified from jelly fig achenes, were subjected to SDS−PAGE and Western blotting (**Figure 1**). Lacking glycosylation, the recombinant PME in *E. coli* was smaller (35 kDa) than the native PME (38 kDa); meanwhile, the recombinant PME harvested from the culture medium of *P. pastori* with an apparent molecular mass of 45 kDa was presumably hyperglycosylated. Despite the difference in their glycosylation, both the native jelly fig PME and the expressed ones were appropriately recognized by the antibodies raised against the native PME.

Activity Staining of the Recombinant PME. To examine if the recombinant PME expressed in yeast was functionally active, two activity staining assays, plate staining and gel staining, were executed using native PME purified from jelly fig achenes as a positive control and the recombinant PME expressed in *E. coli* as a negative control. In the plate staining,

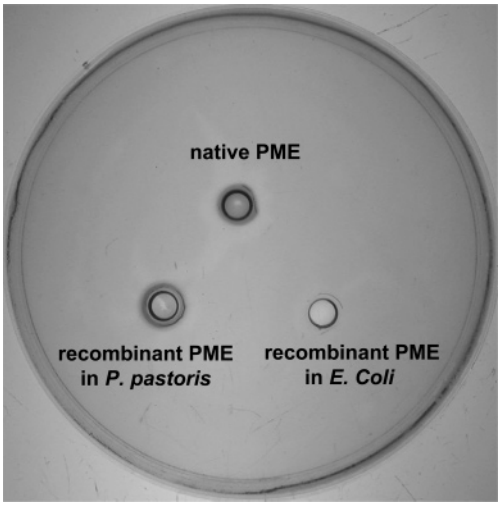

Figure 2. Agarose plate activity staining of native PME and the recombinant ones expressed in *P. pastoris* or *E. coli*.

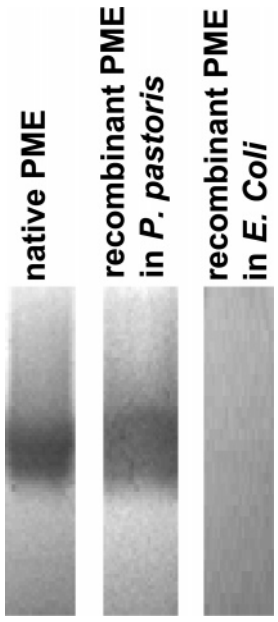

Figure 3. Gel activity staining of native PME and the recombinant ones expressed in *P. pastoris* or *E. coli*.

the activity ring was observed in the recombinant PME expressed in yeast as well as in native PME, but not in the recombinant PME expressed in *E. coli* (**Figure 2**). In gel staining, the activity band was stained to the corresponding protein band of native PME or the recombinant PME expressed in yeast, but not to that of the recombinant PME expressed in *E. coli* (**Figure 3**). Apparently, jelly fig PME was functionally expressed in *P. pastoris*.

Comparison of Enzymatic Activities of the Recombinant and Native PME. To evaluate the quality of the recombinant PME hyperglycosylated in yeast, the enzymatic activity of demethoxylation was quantified and compared between the recombinant and native PME. Enzymatic activity of the recombinant PME was shown to be similar to that of native PME, regardless of the difference in their glycosylation (**Figure 4**). Further analyses indicated that the recombinant PME retained the unique enzymatic activity over a broad pH range found in the jelly fig PME (**Figure 5**). Similar to that of native PME, the optimal pH for enzymatic activity of the recombinant PME was around pH 6. Moreover, both the recombinant PME and

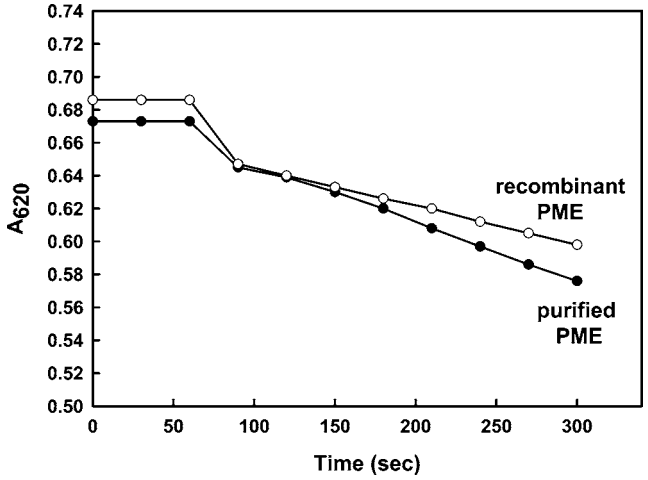

Figure 4. Enzymatic activity assay of native PME and the recombinant PME expressed in *P. pastoris*. Pectin (2 mL) was mixed with 0.15 mL of bromothymol blue and 0.83 mL of water, and the initial A_{620} was recorded. The reaction was started by the addition of PME.

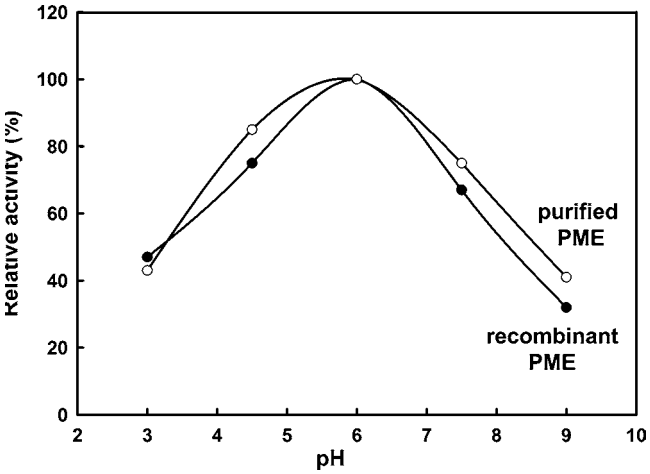

Figure 5. Effect of pH on the enzymatic activity of native PME and the recombinant PME expressed in *P. pastoris*.

native one were thermostable and retained substantial activity up to 65 °C for several hours (**Figure 6**). The recombinant PME, like the native jelly fig PME, was extremely stable, i.e., maintaining full activity at room temperature for several days or at 4 °C for several months (data not shown).

DISCUSSION

The cloudiness of citrus juice and functional properties of commercial citrus pectin (extracted from peel) are related to the degree of methoxylation (*14*), molecular weight (*15*), and intramolecular spatial distribution of methyl esters within the population of pectin molecules (*16*−*18*). Therefore, PME, an enzyme responsible for pectin demethoxylation, is of critical importance in the citrus and pectin industries. As the currently available PMEs are alkaline enzymes without activity in acidic surroundings, juice products have to be titrated to alkaline pH prior to demethoxylation of pectins by this enzyme. Notwithstanding, the acidic PME from jelly fig achene is functionally active over a broad pH range, particularly in acidic environments, and thus can be used in juice products without pH titration. Taken together with its thermostability up to 65 °C, the jelly fig PME is apparently a suitable enzyme for the above commercial purposes as well as other applications, such as producing modified pectins for various utilizations (*19*).

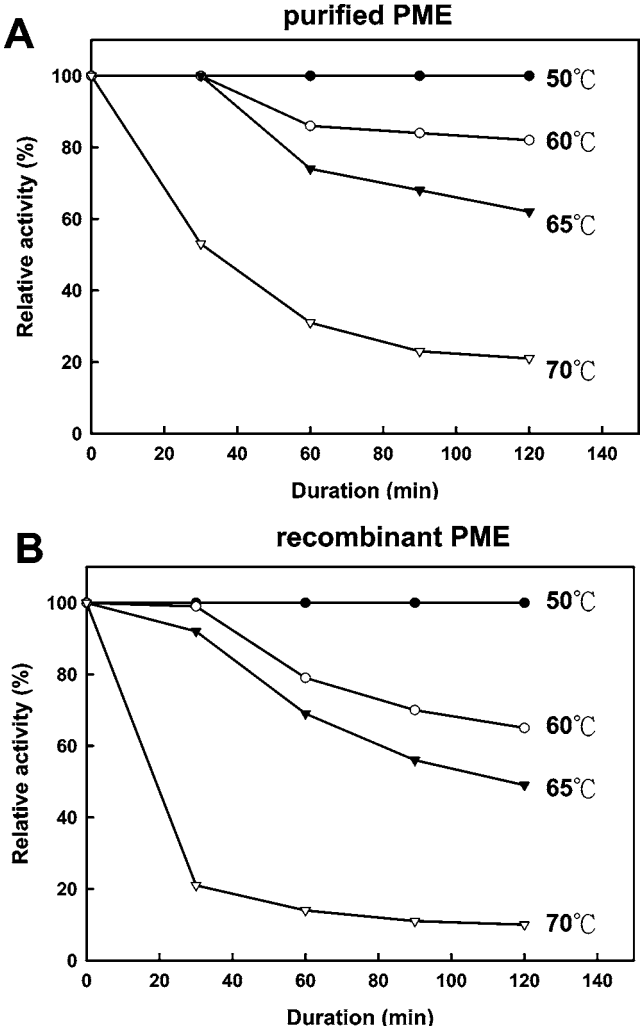

Figure 6. Theromostability of (**A**) native PME and (**B**) the recombinant PME expressed in *P. pastoris*.

Jelly fig is a unique wild plant in Taiwan, and so far most of the jelly fig achenes in the market, relatively expensive ($100/kg at the present time), have been harvested from the wild in the mountain areas (*20*). Cultivation of jelly fig plants in farms has been attempted for the past two decades. Unfortunately, more than 95% of the growers had to give up their cultivation because the jelly fig plants failed to bear mature fruits due to a lack of the symbiotic insect *Blastophaga pumilae* Hill. In addition, the comparatively low abundance of PME in achenes (1−10 mg of PME in 10 g of achene depending on the varieties and growth conditions) has made a direct extraction of this enzyme economically ineffective for commercial purposes. In this study, we successfully expressed jelly fig PME in *P. pastoris* as a secretory protein with enzymatic properties equivalent to those of the native PME. In a larger scale manufacture, we have also successfully produced active jelly fig PME in *P. pastoris* using 5 and 75 L jar fermenters under high-density fermentation. This operation significantly reduced the cost for producing the acidic jelly fig PME. In a pilot run test, 110 g of crude proteins containing 30 g of recombinant jelly fig PME was harvested from a 70 L fermentation. The crude proteins containing recombinant PME have been used directly to reduce the degree of methoxylation of pectin that will be utilized in the food and cosmetic industries in local markets. Moreover, we are currently working on the utilization of the recombinant PME, together with polygalacturonase and pectate lyase, to generate various pectin fragments that possess antibacterial capacity.

In plants, it has been shown that N-linked glycans significantly influence the conformation, stability, and biological activity of glycoproteins (*21*). The native PME isolated from jelly fig achenes has been determined as a N-linked glycoprotein (*4*). While the recombinant PME expressed in *E. coli* lacked glycosylation and enzymatic activity, the recombinant PME expressed in *P. pastoris* was hyperglycosylated and functionally active. Probably, glycosylation is essential for the folding and/or enzymatic activity of jelly fig PME. Sequence analysis indicates the presence in the jelly fig PME of only one potential N-linked glycosylation site (Asn375), right next to the conserved PME motif (residues 376−385) comprising the active site of the enzyme. Whether the N-linked carbohydrate of jelly fig PME is involved in protein folding or interaction with its pectin substrate remains to be studied. In our follow-up research, glycosylations of native jelly fig PME and the recombinant one will be analyzed by mass−mass spectrometry.

ABBREVIATION USED

PME, pectin methylesterase.

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