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Heterologous Expression of Polygalacturonase Genes Isolated from *Galactomyces citri-aurantii* IJ-1 in *Pichia pastoris*

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The objective of this work was to isolate the polygalacturonase genes of *Galactomyces citri-aurantii* IJ-1 harvested from rotten citrus peels and to heterologously express these genes in *Pichia pastoris*. Two polygalacturonase (PG) genes from *G. citri-aurantii* IJ-1 were obtained and tentatively named PG1 and PG2. The genes were cloned into pPICZαC, and expressed in *Pichia pastoris* strain GS115 with a native signal peptide or the α-factor secretion signal peptide of *Saccharomyces cerevisiae*. All of the recombinant proteins were successfully secreted into the culture media and confirmed as a single band with a molecular weight of 35 to 38 kDa by SDS-PAGE. The specific enzyme activities of recombinant PG1 and PG2 purified by His-tag affinity resin were 4,749 and 6,719 U/mg, respectively, with an optimal pH and temperature of pH 4.0 and 50°C. The Michaelis-Menten kinetic constants for PG1 and PG2, K_m, were confirmed to be 0.94 and 0.84 mM, respectively. In the presence of Mn^{2+}, the activity of PG1 and PG2 were increased to 160.8 and 146.4% of normal levels, respectively. In contrast, Cu^{2+} and Fe^{3+} acted as strong inhibitors to the PGs.

Keywords: polygalacturonase, *Galactomyces citri-aurantii*, *Pichia pastoris*, heterologous expression

Introduction

Galactomyces citri-aurantii is one of the major agents responsible for the decay of citrus fruits (Hershenhorn *et al.*, 1992). Plant pathogenic fungi produce highly active hydrolytic enzymes such as pectinase, protease, and xylanase, which are responsible for the degradation of plant cell wall components during invasion by fungi.

Pectinolytic enzymes or pectinases are broadly known as enzymes that cleave pectin substances (Saitou and Nei, 1987). They have been used in various fields, such as the industrial processing of fruits, wastewater treatment, and textile industries (Saitou and Nei, 1987; Baily and Pessa, 1990; Tamura *et al.*, 2007). Among the pectinolytic enzymes, polygalacturonase degrades the α-1,4-linked bonds between two galacturonic acid residues of pectin substances (Miller, 1959). They are classified as endo- and exo-polygalacturonases. The endo-polygalacturonase hydrolyzes polygalacturonic acid in a random fashion and produces oligosaccharidic chains of variable length, whereas exo-polygalacturonase catalyzes single galacturonic acid residues starting from the non-reducing end. Polygalacturonases are included in the glycoside hydrolase (GH) family 28 (Bradford, 1976). Because polygalacturonase is an important enzyme in the food industry, it has been expressed in *Aspergillus niger* (Cereghino and Cregg, 2000), *Saccharomyces cerevisiae* (Beall *et al.*, 1998), *Pichia pastoris* (Liu *et al.*, 2003), and *Bacillus subtilis* (Guo and Ma, 2008).

P. pastoris is an excellent, frequently-used host system for basic research and industrial applications. The *P. pastoris* expression system has two alcohol oxidase genes (*AOX*1 and *AOX*2), which have a strongly inducible promoter and use methanol as a carbon source. *P. pastoris* also has a high growth rate, good protein production rate, and performs eukaryotic posttranslational modifications. Therefore, a number of proteins have been heterologously expressed in this species of yeast (Fogarty and Kelly, 1983).

In this study, we cloned the polygalacturonase genes of *G. citri-aurantii*, which have high pectinase activities, and expressed them in *P. pastoris*. We also analyzed the effects of the native signal peptide of the PGs from *G. citri-aurantii* IJ-1 in *P. pastoris*.

Materials and Methods

Strains, culture media, and plasmids

Escherichia coli DH5α and the pGEM®-T Easy plasmid vector (Promega, USA) were used for cloning. This strain was grown in LB medium, supplemented with ampicillin (100 μg/ml) or zeocin (50 μg/ml) for plasmid selection. *P. pastoris* strain GS115 and the pPICZαC plasmid vector (Invitrogen, USA) were used for PG gene expression. This strain was grown in YPD medium and supplemented with zeocin (100 μg/ml) for the selection of transformants.

Isolation and identification of microorganisms from citrus peel

To isolate microorganisms, we purchased citrus peels from a grocery store in Anseong, Korea. The purchased citrus peels were stored at RT for two weeks, after which rotten citrus peels were selected and suspended in distilled water.

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Table 1. Oligonucleotides used in this study

Primers	Oligonucleotide sequence (5′ to 3′)	References or source
Gal-PG-F	AAGAMSAAGCCMAAGTTC	Li *et al.* (2004)
Gal-PG-R	CCGTTCTYGTARTCYTGY	Li *et al.* (2004)
Gal-PG-full-F	ATGCTTTTYAMHYCYGC	Degenerate primer forward
Gal-PG-full-R	TTARAKKYTGCAAGTRGCAS	Degenerate primer reverse
PG1-*Asu*II-F	AATTTCGAAACGATGCTTTTTTCTACACCT	This work
PG1-*Xba*I-R	AATTCTAGACGAATTCTGCAAGTGGC	This work
PG2-*Asu*II-F	AATTTCGAAACGATGCTTTTTTCTAAATCT	This work
PG2-*Xba*I-R	AATTCTAGACGAATGCTGCAAGTGGC	This work
PG1-2-*Eco*RI-F	AATGAATTCAGCTCCTACTGAAGGTG	This work
5′-*AOX*I	GACTGGTTCCAATTGACAAGC	Invitrogen
3′-*AOX*I	GCAAATGGCATTCTGACATCC	Invitrogen

The suspension was then spread onto YPD agar plates and incubated at 30°C for two days. To identify the isolated fungal strains, we extracted and purified the genomic DNA of the isolated fungi using the Wizard® Genomic DNA Extraction Kit (Promega). The 26S rDNA D1/D2 region was amplified from crude DNA extract and forward and reverse primers (forward primer NL1: 5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′ and reverse primer LR6: 5′-CGC CAG TTC TGC TAC C-3′). The PCR reaction was carried out using a PCR System 2700 (Applied Biosystems,

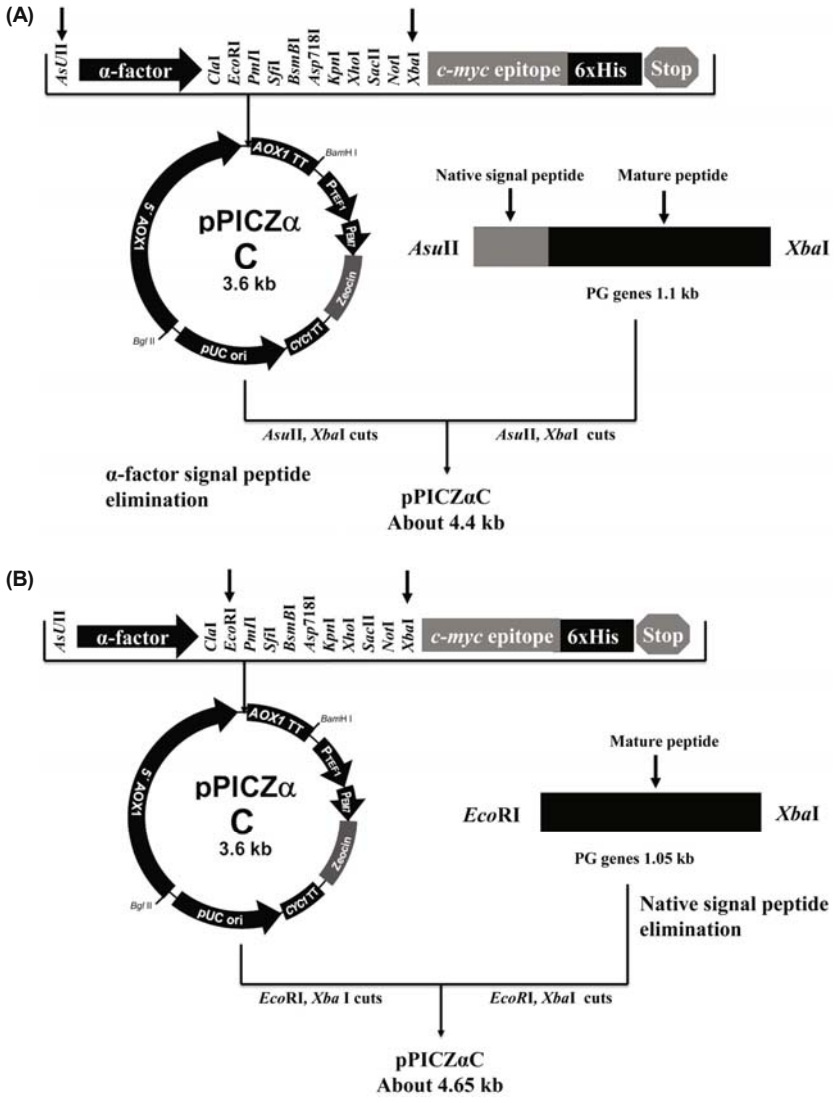

Fig. 1. **Vectors constructed for the expression of polygalacturonase in *P. pastoris*.** (A) The expression vector with native PG signal sequence of *G. citri-aurantii*. (B) The expression vector with the α-factor secretion signal sequence.

USA), under the following conditions: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The analyzed 26S rDNA D1/D2 region sequences were compared with sequences in the NCBI GenBank database using the Blast Network Service. A phylogenetic tree was constructed using the neighbor-joining method to produce a unique final tree under the principle of minimum evolution (Bussink *et al.*, 1990) in the MEGA4 program (Jayani *et al.*, 2005).

Polygalacturonase activity assay

The activity of polygalacturonase was estimated using polygalacturonic acid as the substrate following the method described by Whitaker (1984) and Henriksson *et al.* (1999). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of D-galacturonic acid per min under the assay conditions. The protein concentration was determined according to the procedure described by Bradford using bovine serum albumin as the standard (Markovic and Janecek, 2001).

Isolation of the polygalacturonase gene from cDNA

Polyadenylated RNA was isolated from the total RNA using an mRNA purification kit (Oligotex mRNA Mini Kit, QIAGEN, Germany). The cDNA was synthesized using Maxime RT-premix (iNtRON Biotechnology, Korea) from polyadenylated RNA. Two degenerate oligonucleotides (*Gal*-PG-F and *Gal*-PG-R) corresponding to the highly conserved regions of six fungal PG proteins (*Sclerotinia sclerotiorum* SSPG1, L12023; *B. cinerea* BCPG1, U68715; *Penicillium griseoroseum* PGPG1, AF085238; *F. oxysporum* FOPG, AF078156; and *Aspergillus oryzae* AOPGB, AB00 7769) were synthesized (Table 1). The DNA fragments were amplified from the cDNA by PCR and cloned into the pGEM-T Easy vector (Promega). The sequences of the amplified DNA fragments were analyzed by Macrogen (Korea) and compared to sequences in the NCBI GenBank database using the Blast Network Service Search. Two other degenerate oligonucleotides, corresponding to the N-terminal and C-terminal regions of highly similar PG proteins, were synthesized: *Gal*-PG-Full-F and *Gal*-PG-Full-R (*Galactomyces geotrichum* S31pg2; *Galactomyces geotrichum* GS31pg1; *Geotrichum klebahnii* SE3; and *Galactomyces* geotrichum Ap2PG1) (Table 1). Fragments were amplified from cDNA by PCR and cloned into the pGEM-T Easy vector (Promega) and analyzed by Macrogen.

Potential glycosylation sites of the PGs were predicted using NETNGlyc (www.cbs.dtu.dk/services/NetGlyc).

Construction of the expression vectors

The primers used for construction of the expression vectors with the native PG secretion signal peptide of *G. citri-aurantii* and α-factor secretion signal peptide of *Saccharomyces cerevisiae* were as follows: PG1-*Asu*II-F, PG1-*Xba*I-R, PG2-*Asu*II-F, and PG2-*Xba*I-R (Table 1). PCR was performed with one cycle of 94°C for 10 min, followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 60°C), and extension (1 min 30 sec at 72°C), and a final extension of 72°C for 5 min.

In the expression vector with the native PG signal peptide of *G. citri-aurantii*, the PCR product and expression vector pPICZαC were digested with *Asu*II and *Xba*I (pPICZαC-n/PG) (Figs. 1A and 1B). For insertion into the expression vector pPICZαC with the α-factor signal peptide, the PCR fragments were digested with *Eco*RI and *Xba*I (pPICZαC -α/PG) and cloned. After transformation into *E. coli* DH5α, transformants were selected on LB plates containing 25 μg/ml zeocin. The plasmid was confirmed by PCR with the corresponding primer, restriction enzyme analysis, and DNA sequencing.

Transformation and screening of *P. pastoris* expression strains

The vectors of pPICZαC-n/PG and pPICZαC-α/PG were linearized with *Pme*I, and subsequently transformed into competent *P. pastoris* GS115 cells using an Easyject Plus® (Equibio, Belgium) under the conditions of 1.5 kV, 40 uF, and 125 Ω. The transformed cells were immediately diluted with 1.0 ml of 1.0 M ice-cold sorbitol and were grown on YPD plates (YPD with 1 M sorbitol) containing 50 μg/ml zeocin at 30°C. Only zeocin-resistant transformants survived and grew normally after four days. The obtained transformants were confirmed by genomic PCR assay using 5′*AOX*I and 3′*AOX*I primers.

Expression of polygalacturonase genes

Transformants were incubated in 50 ml of BMGY (1% yeast extract, 2% peptone, 1.34% YNB, 1% glycerol, 0.00004% biotin, and 100 mM of potassium phosphate buffer, pH 6.0), and shaken (200 rpm) at 30°C until cultures reached an OD_{600} value of 0.6. The cells were harvested by centrifugation at 3,000×g for 10 min at room temperature. The cell pellet was resuspended in 50 ml of BMMY induction medium (identical to BMGY, except for containing 1% methanol instead of 1% glycerol) and cultured at 30°C for six days with shaking at 200 rpm. Methanol was added to a final concentration of 0.5% (v/v) every 24 h. The expression of polygalacturonase in the culture supernatants was monitored at 1, 2, 3, 4, 5, and 6 days by measuring the expressed recombinant PG activity.

Purification of recombinant proteins

The recombinant proteins were subjected to His-tag purification using a gravity column that was packed with nickel-NTA agarose (QIAGEN). After the Ni^{2+}-NTA slurry was passed through the column, it was equilibrated with five column volumes of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). Supernatants of the expressed recombinant strains were applied to the equilibrated column. The weakly bound proteins were washed from the column using five column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0). The recombinant proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0) at 0.5 ml/min.

Analysis of N-terminal amino acids of recombinant proteins

For N-terminal sequencing, the proteins were electrotrans-

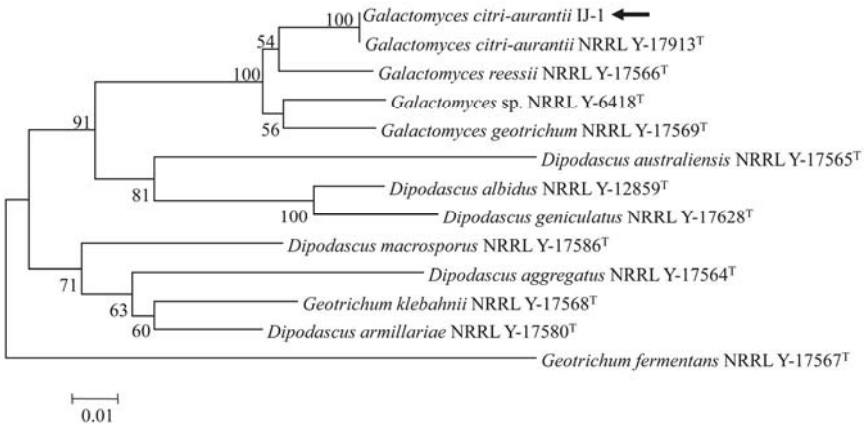

Fig. 2. Neighbor-joining phylogenetic tree based on 26S rRNA gene sequences showing the position of strain IJ-1.

ferred to a polyvinylidene difluoride (PVDF) membrane using the Mini Trans-Blot® Cell (Bio-Rad, USA), and the appropriate PVDF band was cut and subsequently sequenced by the Edman degradation method using a Procise® 492cLC protein sequencer (Basic Science Institute, Korea).

Optimal pH and temperatures of recombinant protein

The optimal pH of the recombinant PGs was assayed in 50 mM acetate buffer (pH 3–5), citrate buffer (pH 3–5), citrate-phosphate buffer (pH 3–8), and phosphate buffer (pH 6–8) at 37°C. To determine the optimal temperature, PG activity was determined between 20 and 80°C at the optimum pH. We used 0.5% polygalacturonic acid (Sigma) as the substrate.

Effects of temperature, pH, and metal ions

For thermostability determination, the enzyme was incubated at 0, 20, 30, 40, 50, and 60°C. The enzyme was extracted every 30 min for 150 min and residual activity was assayed. To evaluate the effect of pH on PG activity, 50 mM citrate (pH 3–5), 50 mM phosphate (pH 6–8), and 50 mM carboxylate (pH 9–11) buffers were used. The enzymes were treated with different buffers for 5 h at 0°C.

To determine the effects of various metal ions, enzymes were incubated with 1 mM concentrations of either Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} for 1 h at 0°C. After treatments using different temperatures, pHs, and metal ions, the samples were assayed for 30 min at 30°C in 50 mM sodium phosphate buffer (pH 6.0). The enzyme in the absence of each parameter was expressed as the 100% activity level.

Enzyme kinetics

The K_m and K_{cat} constants for enzyme kinetics were determined at different concentrations of substrate (from 0.1 to 0.5%). The enzyme was assayed at various concentrations for 30 min at 30°C in 50 mM sodium phosphate buffer (pH 6.0). The kinetic data were calculated and defined by the Michaelis-Menten equation.

Results

Isolation and identification of microorganisms with high polygalacturonase activity

To select microorganisms with high polygalacturonase activity, we incubated strains isolated from rotten citrus peels in YPD culture media at 30°C for 30 h and the polygalacturonase activity was analyzed for each supernatant. We used 26S rDNA sequences to identify the strain having the

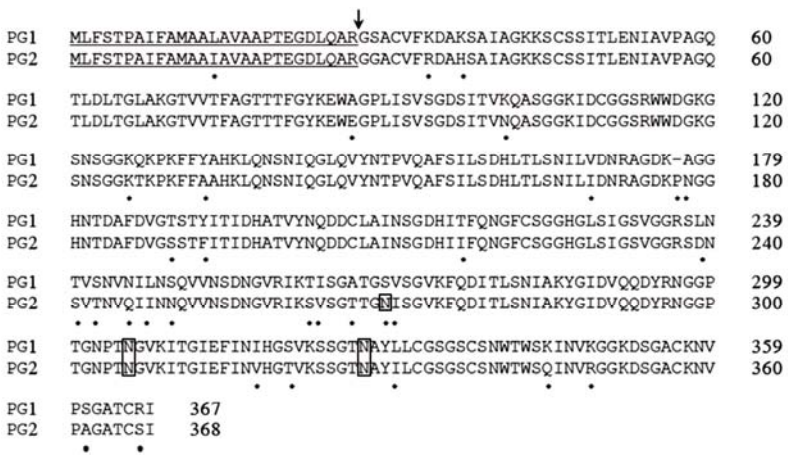

Fig. 3. The deduced amino acid sequences of PG1 and PG2. Underline: signal peptides. *, mismatched amino acid residues of PG1 and PG2; Arrow, signal peptide cleavage site; Box, potential N-glycosylation sites.

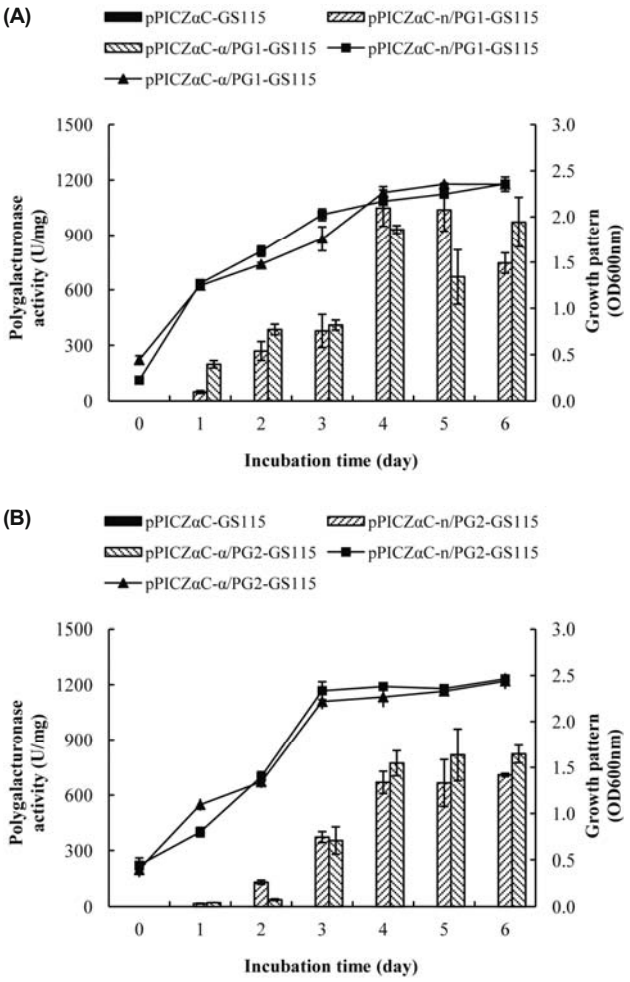

Fig. 4. Comparison of PG expression in *P. pastoris* GS115 with the native or the α-factor secretion signal peptides. The data represent the mean of three independent experiments. Line and bar graphs are the growth pattern and enzyme activity, respectively.

highest polygalacturonase activity (data not shown), and found that it had a greater than 99% homology with *Galactomyces citri-aurantii* NRRL Y-17913^{T}. This strain was tentatively named *G. citri-aurantii* IJ-1 (Fig. 2).

Isolation of the polygalacturonase genes of *G. citri-aurantii* IJ-1

The polygalacturonase (PG) genes of *G. citri-aurantii* IJ-1 were partially amplified from cDNA with PCR primers (*Gal*-PG-F and *Gal*-PG-R) that were designed based on the conserved regions among fungal PG genes (Table 1). The amplified fragment had 99% homology with the endo-polygalacturonase amino acid sequences of *G. geotrichum*. With conserved primers (*Gal*-PG-full-F and *Gal*-PG-full-R) (Table 1) that were newly designed to isolate the complete coding sequences of the PG genes of *G. citri-aurantii* IJ-1, we amplified two genes, which we named PG1 and PG2. The PG1 and PG2 sequences were 92.21% similar. The translated amino acids of PG 1 and PG2 obtained by the NCBI Open Reading Frame (ORF) Finder were 367 and

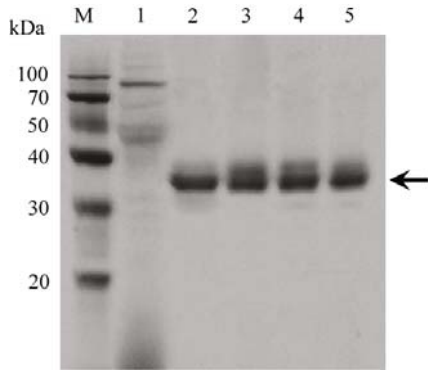

Fig. 5. SDS-PAGE analysis of purified recombinant PG1 and PG2. Lanes: M, standard protein molecular weight markers; 1, pPICZαC without PG gene; 2, pPICZαC-n/PG1-GS115 with native signal sequence; 3, pPICZαC-α/PG1-GS115; 4, pPICZαC-n/PG2-GS115 with native signal sequence; lane 5, pPICZαC-α/PG2-GS115.

368 amino acid residues, respectively (Fig. 3). In an analysis of the amino acid sequences, PG1 and PG2 were 98% identical to the protopectinase (*SE3*) and endo-polyglacturonase genes (*S31PG1*) of *G. klebahnii*, respectively. The GenBank accession numbers for PG1 and PG2 gene sequences are JQ337943 and JQ337944, respectively.

Expression of polygalacturonase genes in *P. pastoris*

We expressed the PG genes of *G. citri-aurantii* IJ-1 in *P.*

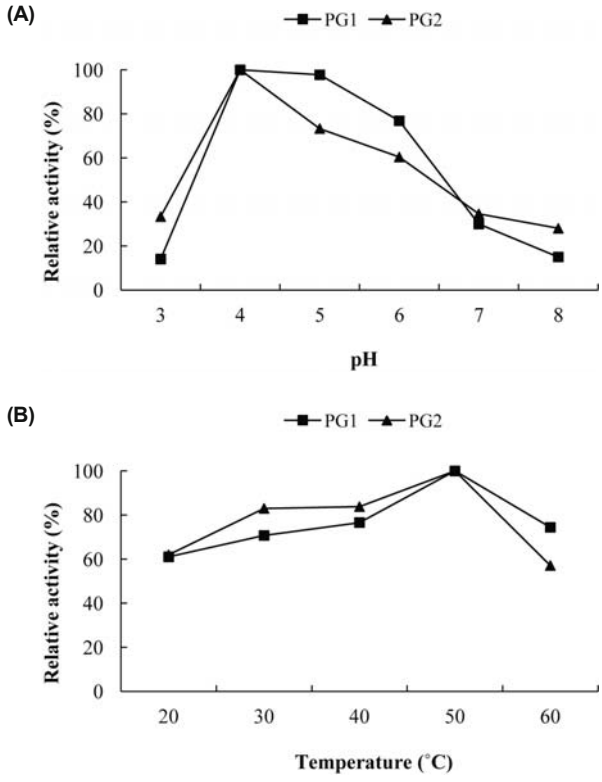

Fig. 6. Optimal pH (A) and temperature (B) of recombinant PGs. The data represent the mean of three independent experiments.

Table 2. Effect of metal ions on the activity of PGs

Metal ions (1 mM)	Residual activity (%)[a]	
	PG1	PG2
Control	100	100
Ca^{2+}	60.8±0.6	63.2±1.7
Co^{2+}	106.4±2.1	92.2±0.6
Cu^{2+}	16.8±0.9	34.4±2.6
Fe^{3+}	9.4±0.1	10.4±1.2
Mg^{2+}	103.5±0.6	100.3±0.3
Mn^{2+}	160.8±2.1	146.4±1.7
Zn^{2+}	71.8±1.2	71.6±0.8

[a] Values are means±SD for these different experiments.

pastoris GS115 using expression vectors containing two different signal peptides, the native PG secretion signal peptide of *G. citri-aurantii* and the α-factor secretion signal peptide of *S. cerevisiae* (pPICZαC-n/PG1 and 2; pPICZαC-α/PG1 and 2). The growth patterns and the polygalacturonase activities of the recombinant proteins are shown in Fig. 4. The polygalacturonase activity of the recombinant PG1 protein with the native signal peptide was higher than that with the α-factor secretion signal peptide, whereas the recombinant PG2 protein showed higher activity with the α-factor secretion signal peptide (Fig. 4). The specific activities of the recombinant PG1 and PG2 proteins purified by his-tag affinity resin were 4,749 U/mg and 6,719 U/mg, respectively, and the molecular sizes were between 35 and 38 kDa on SDS-PAGE (Fig. 5). The cleavage site of the signal peptides for the PG1 and PG2 genes of *G. citri-aurantii* IJ-1 was 27 residues from the N-terminus, based on analysis of the N-terminal amino acid sequence (Fig. 3), and several N-glycosylated sites were confirmed.

Optimal pH and temperature of the recombinant proteins

The optimal pH value of the purified recombinant PG1 was between 4 and 5, while that of PG2 was 4 (Fig. 6A). Both proteins retained over 60% of their original enzyme activity between pH 4 and 6. At a neutral pH of 7, only 30% of enzyme activity was observed. This suggests that PG1 and PG2 of *G. citri-aurantii* IJ-1 are acidic enzymes. The optimal temperature for the purified recombinant PG1 and PG2 was 50°C (Fig. 6B). The enzyme activities of both recombinant proteins were stable across temperatures ranging from 20 to 60°C. In this temperature range, over 60% of enzyme activity was retained.

Effect of metal ions on the stability of PGs

The activity of the PGs was assayed in the presence of different metal ions at 1 mM concentrations. Among the various metal ions, Mn^{2+} stimulated the activity of PG1 and PG2 to 160.8 and 146.4%, respectively (Table 2). In contrast, the activity of the PGs was decreased in the presence of Ca^{2+},

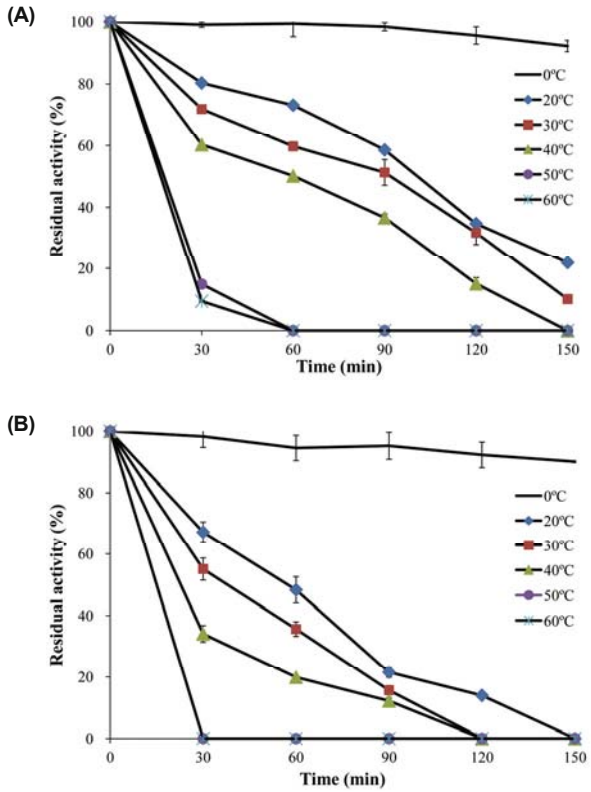

Fig. 7. Thermal stability of the purified PG1 (A) PG2 (B) at 0 (★), 20 (◆), 30 (■), 40 (▲), 50 (●), and 60°C (×). Residual activity was assayed at for 30 min at 30°C in 50 mM sodium phosphate buffer (pH 6.0). The data represent the mean of three independent experiments.

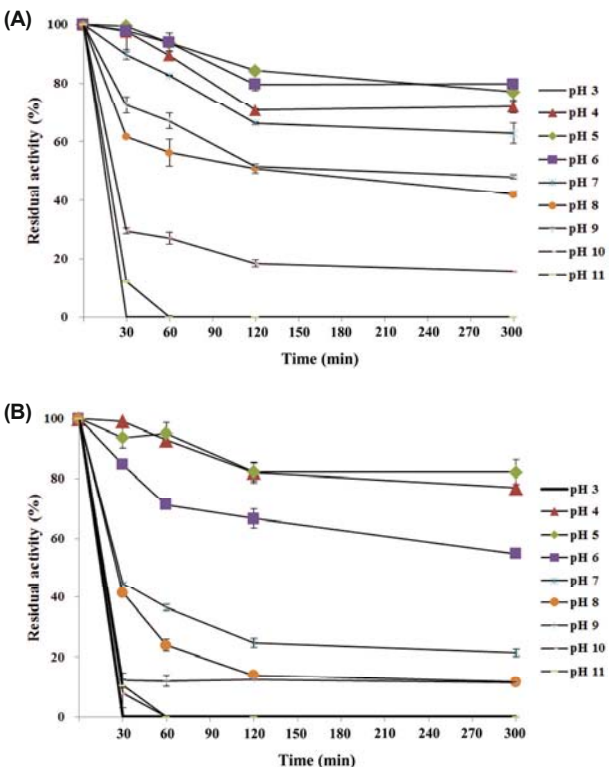

Fig. 8. Effect of pH on stability of the PGs. The enzymes were incubated with different buffers for 5 h at 0°C. 50 mM citrate (pH 3–5), 50 mM phosphate (pH 6–8), and 50 mM carboxylate (pH 9–11) buffers were used. Residual activity was assayed at for 30 min at 30°C in 50 mM sodium phosphate buffer (pH 6.0).

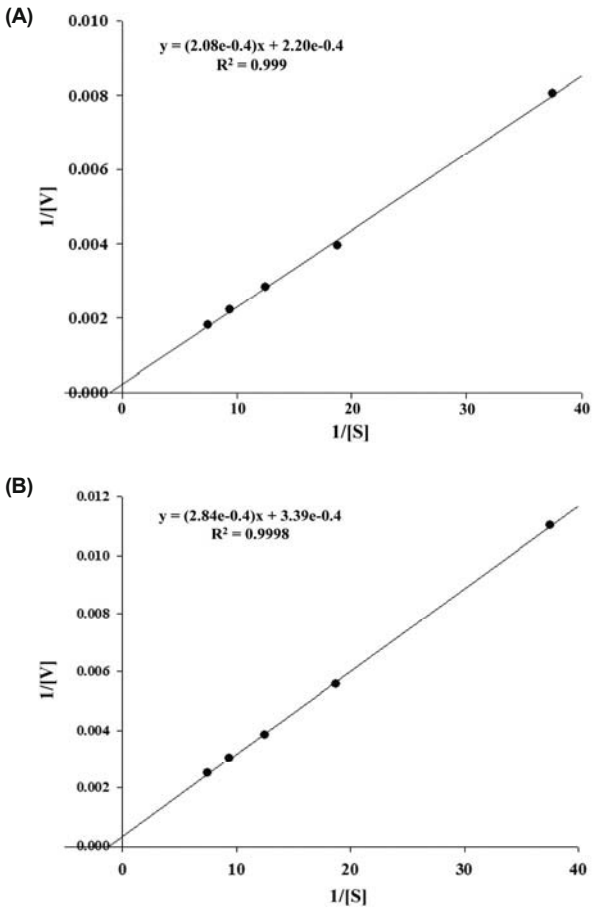

Fig. 9. Double-reciprocal plot for evaluating the kinetics parameters of the PGs. The activity was assayed at different concentrations of substrate for 30 min at 30°C.

Cu^{2+}, Fe^{3+}, and Zn^{2+}. Of these, Fe^{3+} was the strongest inhibitor of the PGs. However, Cu^{2+} and Mg^{2+} had no effect on the activity of the PGs.

Effect of temperature on the stability of PGs

Neither PG1 nor PG2 was thermostable. In the case of the PG1 enzyme, 50% of activity was retained after 60 min at 20 to 40°C (Fig. 7A). However, the activity of PG1 was completely inhibited within 60 min at between 50 and 60°C. It was observed that the PG2 was less stable than PG1 at 20 to 60°C (Fig. 7B). At 50 to 60°C, the PG2 activity was completely inhibited within 30 min and not retained after 150 min at other temperatures.

Effect of pH on the stability of the PGs

Evaluation of the effect of pH on the stability of PGs was conducted at 0°C. Both PGs lost stability under strong acidic (pH 3) and alkali conditions (pH 10 and 11) (Fig. 8). However, PG1 showed more stability than PG2 at various pH ranges. In the case of PG1, over 70% of enzyme activity was retained for 5 h at pH 4 to 7 (Fig. 8A). PG1 was also shown to retain 50% activity at pH 8 and 9. In PG2, the stability was retained at pH 4 to 6 for 5 h (Fig. 8B). However, PG2 showed a decrease in stability, falling to less than 50% activity, at a pH of 7 to 9 within 30 min. In addition, the activity of PG2 was completely inhibited within 60 min under harsh pH conditions (pH 3, 10, and 11).

Table 3. Kinetics characteristics of PGs

Enzyme	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (sec^{-1}/mM^{-1})
PG1	0.94	2881.6	3052.2
PG2	0.84	1867.1	2229.2

Kinetic determination

The kinetic constants of the PGs were evaluated from Lineweaver-Burk plots of the activity at different concentrations of substrate for 30 min at 30°C (Fig. 9).The K_m values of PG1 and PG2 were 0.94 and 0.84 mM, and the K_{cat} values were 2881.6 and 1867.1 sec^{-1}, respectively (Table 3). Kinetic efficiency (K_{cat} /K_m) values were determined to be 3052.2 and 2229.2 sec^{-1}/mM^{-1} for PG1 and PG2, respectively.

Discussion

G. citri-aurantii abundantly secretes endo-polygalactronase during the initial stages of fruit infection, mainly in citrus fruits, as one effective mechanism for survival (Hershenhorn *et al.*, 1992). Most phytopathogenic fungi secrete polygalacturonase at the early stages of infection (de Lorenzo *et al.*, 2001). For successful plant colonization and depolymerization of plant cell-wall components, fungi produce cell wall-degrading enzymes (CWDEs) such as endo- and exo-polygalacturonase, cellulase, hemicellulase, among other enzymes (Carpita and Gibesut, 1993; Annis and Goodwin, 1997).

We isolated *G. citri-aurantii* IJ-1 from citrus fruit harvested in Korea. We isolated and identified two polygalacturonase (PG) genes from *G. citri-aurantii* IJ-1, PG1, and PG2. Each gene was cloned into the *P. pastoris* expression vector, pPICZαC, and expressed in *P. pastoris* strain GS115 with either the native signal peptide of the PG genes of *G. citri-aurantii* IJ-1 or the α-factor secretion signal peptide of *S. cerevisiae*. In the *P. pastoris* expression system, the secretion signal peptide is important and essential for the successful expression of heterologous proteins in *P. pastoris*. For example, for endo-β-1,4-glucanase expression, the expression yield with the native signal sequence is much lower than that with the α-factor secretion signal (Ding *et al.*, 2002). Human monocyte chemoattractant protein-1 was expressed at the same level with either its native signal sequence or the α-factor secretion signal (Lang and Looman, 1995). Lactase activity with the native signal peptide was five-fold higher than that with the α-factor secretion signal (Blanco *et al.*, 2002). The alkaline protease activity with the native signal peptide was 1.5-fold higher than that with the α-factor secretion signal (Hemila *et al.*, 1992). In this study, PG1 and PG2 of *G. citri-aurantii* IJ-1 were successfully expressed in *P. pastoris* using either the native signal peptide of PG or the α-factor signal peptide, although both signal sequences resulted in slightly different enzyme activities.

SDS-PAGE analysis was conducted to evaluate the molecular

weights of the PGs. Both PGs were confirmed to have molecular weights between 35 and 38 kDa (Fig. 5). However, the presence of multiple bands indicating slightly different molecular weights was also observed. As a result of the prediction of the potential glycosylation of the PGs, several N-glycosylated sites were predicted and marked by boxes (Fig. 3). Therefore, purified recombinant enzymes could be post-translationally modified by glycosylation and the multiple smeared bands were assumed to be glycosylated enzymes.

In this study, the specific enzyme activities of the recombinant PG1 and PG2 were 4,749 and 6,719 U/mg, respectively (Fig. 4). When compared with other PGs isolated from various microbial sources, the recombinant PGs had the highest enzyme activities. Many fungal PGs exhibited specific activities of less than 500 U/mg (Barnby *et al*., 1990; Pietro and Roncero, 1996; Singh *et al*., 1999b; Nagai *et al*., 2000; Takao *et al*., 2001), except for the PGs obtained from *Thermococcus aurantiacus*, *Aspergillus niger*, *Penicillium frequentans*, and *Sclerotinia borealis* (Favey *et al*., 1992; Takao *et al*., 2000, 2001; Singh and Rao, 2002). Also, The PGs produced from *Bacillus* sp. KSM-P443 and *B. licheniformis* were reported to exhibit low specific enzyme activities of 54 and 209 U/mg, respectively (Singh *et al*., 1999b; Koboyashi *et al*., 2001). Therefore, our recombinant PGs were successfully expressed and showed one of the highest enzymes activity compared to other PGs produced from different microbial sources.

The optimal pH values of the purified recombinant PG1 ranged from 4 to 5, and for PG2 was 4 (Fig. 6A). Many fungal PGs obtained from *Aspergillus* spp., *Saccharomyces cerevisiae*, and *Sclerotinia sclerotiorum* had optimal pH values in the range of 3.5 to 5.5 (Koboyashi *et al*., 1999; Corredig *et al*., 2000; Singh and Rao, 2002; Hasunuma *et al*., 2003). However, the optimal pH values of PGs produced from *Bacillus* spp. were found to be in the neutral and alkaline ranges (Singh *et al*., 1999b; Koboyashi *et al*., 2001). The optimal temperature of the purified recombinant PG1 and PG2 was 50°C (Fig. 6B). Most of the other fungal PGs were reported to have a similar temperature optimum of 50°C, including *Sclerotinia* spp. and *Saccharomyces* spp., as reported previously (Singh *et al*., 1999a; Corredig *et al*., 2000; Takao *et al*., 2000, 2001). However, some PGs isolated from *Bacillus* spp. and *F. oxysporum* f. sp. *lycopersici* showed the ability to hydrolyze the substrate at higher temperatures (Pietro and Roncero, 1996; Singh *et al*., 1999b; Koboyahsi *et al*., 2001). The recombinant PGs were sensitive to temperature fluctuations. The PG1 and PG2 lost stability within 150 min over a temperature range of 20 to 60°C (Fig. 7), unlike previously reported fungal PGs (Singh *et al*., 1999a; Corredig *et al*., 2000; Takao *et al*., 2001; Singh and Rao, 2002). Their temperature stability was retained between 40 and 75°C. Additionally, the stability of the purified recombinant PGs was retained at pH 4 to 7 (Fig. 8), similar to that of other fungal enzymes. However, the PGs isolated from *Bacillus* spp. showed greater stability under neutral and alkali conditions (pH 7 to 12), unlike other fungal enzymes (Singh *et al*., 1999a; Koboyashi *et al*., 2001).

In the kinetic determination, the K_m values of PG1 and PG2 were 0.94 and 0.84 mM, respectively. The affinity of PG2 toward substrates was higher than that of PG1. The enzyme isolated from the fungi showed a similar K_m value to those of *S. sclerotiorum* and *A. niger* (Koboyashi *et al*., 1999; Singh and Rao, 2002).

In conclusion, when considering the high specific enzyme activity, the substrate affinity, and the synergistic effect with Mn^{2+}, recombinant PGs are candidate acidic enzymes with a wide temperature range for use in the fruit processing, wastewater treatment, and textile industries.

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