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Characterization of an Apple Polygalacturonase-Inhibiting Protein (PGIP) That Specifically Inhibits an Endopolygalacturonase (PG) Purified from Apple Fruits Infected with *Botryosphaeria dothidea*

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Abstract An apple polygalacturonase-inhibiting protein (PGIP), which specifically inhibits endopolygalacturonase (PG, EC 3.2.1.15) from *Botryosphaeria dothidea*, was purified from *Botryosphaeria dothidea*-infected apple (*Malus domestica* cv. Fuji) fruits. The purified apple PGIP had a molecular mass of 40 kDa. The N-terminal amino acid sequence of the purified protein showed high homologies to those of PGIP from pear (100%), tomato (70%), and bean (65%). We also purified polygalacturonase (PG) from *B. dothidea*. The PG hydrolyzes pectic components of plant cell walls. When the extracted apple pectic cell wall material was treated with purified apple PGIP and *B. dothidea* PG, the amount of uronic acid released was lower than that treated with *B. dothidea* PG alone. This result demonstrates that PGIP functions specifically by inhibiting cell wall maceration of *B. dothidea* PG. Furthermore, we characterized the *de novo* function of the PGIP against PG on the solubilization and depolymerization of polyuronides from cell wall of apple fruits inoculated with *B. dothidea*. This result demonstrated that the PGIP of plants exhibits one of the direct defense mechanisms against pathogen attack by inhibiting PGs that are released from pathogens for hydrolysis of cell wall components of plants.

Key words: Polygalacturonase-inhibiting protein (PGIP), polygalacturonase (PG), apple, *Malus domestica*, *Glomerella cingulata*, *Botryosphaeria dothidea*, enzyme assay

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Fungal pathogens produce cell-wall-degrading enzymes that facilitate the invasion of higher plant tissues [7, 10, 13]. These enzymes, such as polygalacturonases (PG, EC 3.2.1.15), glucanases (EC. 3.2.1.6), and pectin-methylesterases (PME, EC 3.1.1.11), degrade structural components of plant cell wall [8, 21, 28]. The PGs from fungal pathogens are important cell-wall-degrading enzymes that hydrolyze pectin and release short-chain galacturonic acids [19, 30, 34–37].

In higher plants, polygalacturonase-inhibiting proteins (PGIPs) are located in the cell wall and directly inhibit the activity of PGs [6, 14, 15, 17, 18, 20, 22, 31]. The PGIP activity from certain higher plant species is usually targeted towards a specific fungal PG, and usually has little or no inhibitory effect on PGs from alternate fungi [1, 18]. This specificity of action has also been observed with various PG isozymes from a single fungal species [18, 30, 38]. For example, PGIP from pear inhibits the PGs only from *Aspergillus niger*, *Botrytis cinerea*, and *Dothiorella gregaria* [1, 9].

The PGIP from Golden Delicious apples was purified [38]. However, the purified apple PGIP did not inhibit the activity of PG isolated from apple fruit inoculated with *B. cinerea*. In this study, we purified a PGIP from Fuji apple fruit inoculated with *B. dothidea*, and the PGIP showed inhibitory activity against a PG from that species. Furthermore, we characterized the activity of PGIP against *Glomerella cingulata*, and were able to examine the catalytic properties of PG purified from apple fruit that was inoculated with *B. dothidea*. Moreover, in this study, we for the first time

demonstrated the *in vivo* function of the PGIP on the solubilization and depolymerization of polyuronides from cell wall of apple fruit.

MATERIALS AND METHODS

Fungal and Plant Materials

B. dothidea and *G. cingulata* were isolated from naturally infected mature apple fruits (*Malus domestica* cv. Fuji). Fuji apple fruits were harvested and stored at 4°C for up to 90 days.

Preparation of Fungal PGs

PGs from *B. dothidea* and *G. cingulata* were purified, essentially as previously described [27]. Apple fruits were washed and then inoculated to a depth of 4 mm with a needle containing a suspension of *B. dothidea* or *G. cingulata* spores at a concentration of 5×10^5 spores/ml. The inoculated apples were placed in plastic boxes and incubated for 7 days at 27±1°C. Five-hundred g of decayed apple fruit was homogenized in 1 l of 10 mM sodium phosphate buffer (pH 7.0) containing 2% (w/w) polyvinylpolypyrolidone. All purification procedures were performed at 4°C. The homogenate was centrifuged at 12,000 ×*g* for 60 min, and ammonium sulfate at 85% saturation was added to the supernatant. The pellet, obtained by centrifugation as described above, was dissolved in 100 ml of deionized H_2O. The resultant solution was dialyzed against 2 l of 10 mM sodium phosphate buffer (pH 7.0) for 48 h with stirring, and the dialysate was centrifuged at 12,000 ×*g* for 60 min. The resultant supernatant was concentrated using an Amicon Diaflo System with PM-10 membrane (Millipore, Billerica, MA, U.S.A.). Concentrated crude extracts were loaded onto a 2.8×60 cm column of Sephadex G-100 (Sigma-Aldrich, St. Louis, MO, U.S.A.) and were eluted with 10 mM sodium phosphate buffer (pH 6.0). The PG activity of 5 ml each fraction was measured as described below. Fractions containing PG activity were pooled and concentrated as described above. The concentrated samples were applied to a 2.8×50 cm column of DEAE-Cellulose (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 10 mM Tris-HCl buffer (pH 7.2), or a 1.5×20 cm column of QAE-Sephadex A-50 (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 10 mM Tris-HCl buffer (pH 8.0), and proteins were eluted with a linear gradient formed from 0 to 1.0 M NaCl in the same buffer.

Purification of Apple PGIP

Apple PGIP was purified as previously described [38]. All purification steps were performed at 4°C. Diced apple fruit (1 kg) was mixed with solid 1.5% (w/w) polyvinylpolypyrolidone and 0.2% (w/w) sodium bisulfate, and the resultant slurry was homogenized in 2 l of 50 mM sodium acetate buffer (pH 6.0). The homogenate was filtered through 3 layers of Miracloth (Calbiochem-Behring, La Jolla, CA, U.S.A.) and the filtrate was washed three times with 1 volume of deionized water. After washing, the insoluble tissue was resuspended in 1 l of 50 mM sodium acetate containing 0.2% sodium bisulfate, pH 6.0, and NaCl was added to a final concentration of 1.0 M. The resuspended sample was stirred for 30 min at 4°C and was centrifuged at 12,000 ×*g* for 60 min at 4°C as described above. The supernatant was dialyzed against resolubilization buffer and was concentrated using an Amicon Diaflo System with PM-10 membrane. The concentrated crude extract was loaded onto a 2.8×60 cm column of Sephadex G-100 and was eluted with 10 mM sodium phosphate buffer (pH 6.0). PGIP activity was measured in 5 ml of each fraction. Fractions containing PGIP activity were pooled, concentrated using Amicon Diaflo System PM-10 membrane, and then applied to a 1.5×20 cm column of CM-Sephadex C-50 in 10 mM sodium phosphate buffer (pH 6.0). Fractions containing PGIP activity were then pooled, concentrated, and applied to a 1.5×20 cm column of QAE-Sephadex A-50 (Sigma, St. Louis, MO, U.S.A.) in 10 mM Tris-HCl buffer (pH 8.0). Proteins were eluted with a linear gradient formed from 0 to 1.0 M NaCl in the same buffer. The fractions containing PGIP activity were then pooled, concentrated, and applied onto a Sephacryl S-200 column (1.5×90 cm), and the column was eluted with 10 mM sodium phosphate buffer (pH 6.0). Active fractions (2 ml) were pooled and concentrated as described above. Protein concentration was measured by using the modified Bradford method [4].

Enzyme Assay

PG activity was measured, as the increase of reducing sugars, with 2-cyanoacetamide as described by Gross [16, 35]. The reaction mixture consisted of 100 µl of 0.4% polygalacturonic acid in 100 mM sodium acetate (pH 5.5) and 100 µl of enzyme suspension. The reaction mixture was incubated for 1 h at 30°C, and the reaction was then terminated by addition of 1 ml of cold 100 mM borate buffer (pH 9.0), and 200 µl of 1% 2-cyanoacetamide. The reaction was terminated by boiling in a water bath for 10 min. The change in absorbance at 276 nm was measured after cooling. One unit of PG activity was defined as the amount of galacturonic acid released at a rate of 1 µmol/min at 30°C. The inhibition of PG activity by PGIP was determined as rates of sugar production from polygalacturonic acid in the presence or absence of PGIP [38]. The reaction mixture consisted of 100 µl of 0.4% polygalacturonic acid in 100 mM sodium acetate (pH 5.5), 50 µl of PG enzyme suspension, and in the presence or absence of 50 µl of PGIP. After 1 h of incubation at 30°C, the reaction was terminated by the addition of 1 ml of cold 100 mM borate buffer (pH 9.0) and 200 µl of 1% 2-cyanoacetamide. This mixture was placed in a boiling water bath for 10 min. After cooling, absorbance was measured as described

above. One unit of PGIP was defined as the amount of inhibitor required to reduce the activity of 1.5 unit of *B. dothidea* PG by 50%.

N-Terminal AminoAcid Sequence of the Purified Apple PGIP

Proteins were fractionated by 12% SDS-PAGE with 4.5% stacking gel as described previously [23]. The gels were stained with a Bio-Rad Silver stain kit (Bio-Rad, Hercules, CA, U.S.A.). Amino acid sequences were analyzed as previously described [26]. After resolving the purified PGIP by SDS-PAGE, protein bands were electroblotted onto PVDF membranes (Millipore, Billerica, MA, U.S.A.) in 10 mM 3-(cyclohexyamino)-1-propane-sulfonic acid buffer containing 10% methanol (pH 11.0). Analysis of the amino acid sequence was performed on an Applied Biosystems 494 protein sequencer (ABI Inc., Foster City, CA, U.S.A.) at the Protein Facility of Iowa State University, Ames, IA, U.S.A.

Cell Wall Extraction

Cell wall materials were prepared as described by Conway *et al.* [8]. Apple fruits (200 g) were homogenized in 80% ethanol, and the homogenate was incubated at 80°C for 10 min to inactivate enzyme activity. The suspension was filtered through a Miracloth and washed 3 times with 80% ethanol. The residue was immediately transferred to 20 mM HEPES-NaOH buffer (pH 6.0) and filtered twice through a Miracloth. The residue was suspended in 30 ml of 20 mM HEPES-NaOH buffer (pH 6.0) and 30 µl of α-amylase (Type-A, Sigma, St. Louis, MO, U.S.A.), and two drops of toluene were added. This mixture was incubated at 30°C for 24 h with gentle shaking and then filtered through a Miracloth. The residue was washed successively with phenol/acetic acid/H_2O (2:1:1, w/v/v), chloroform-methanol (1:1, v/v), and acetone for 10 min each with shaking, and collected by filtration through a Miracloth. Each of these washing steps was repeated twice. The dried cell wall residue was stored in a desiccator until use.

Extraction of Polyuronides

Polyuronides from 100 mg of cell wall were extracted successively with distilled water, 50 mM 1,2-diaminocyclohexane tetra acetate (CDTA) in potassium phosphate buffer (pH 6.5), and 50 mM Na_2CO_3 in 20 mM $NaBH_4$, as previously described [29]. The extracts were designated as water-soluble CDTA and Na_2CO_3 fractions, respectively.

Enzyme Treatment of the Extracted Polyuronides and Gel Chromatography

Enzyme treatment was performed as previously described [39, 40]. Twenty mg each of the cell wall, water-soluble, CDTA, and Na_2CO_3 fractions was suspended in 2 ml of reaction mixture consisting of 100 mM sodium acetate (pH 5.5) containing 50 units of *B. dothidea* PG. Forty units of the purified PGIP was added to each fraction, and the mixtures were incubated for 24 h at 30°C. Each reaction mixture was applied to a Sepharose CL-2B column (2×99 cm), which was preequilibrated with 50 mM sodium acetate buffer (pH 6.0) containing 100 mM NaCl, and every 2.5 fractions was collected. Total carbohydrates in each column fraction was measured using the phenol-sulfuric acid method [12], and uronic acid content was measured using the carbazole method [2].

Analysis of Neutral Sugar Contents

Cell wall material was hydrolyzed with 2 N trifluoroacetic acid at 121°C for 1 h, and the resulting hydrolysates were converted to alditol acetates [3]. Neutral sugar contents were analyzed using a gas chromatograph (model HP 5890) with a 30 m×0.32 mm SP-2380 capillary column using the following condition: injection temperature 270°C, column temperature 220°C, detector temperature 270°C, using a He carrier gas at 24.5 ml/min.

RESULTS

Purification and Inhibition Specificity of Apple PGIP

Three PGs were purified, and their activities on apple tissue with PGIP from apple fruit infected with *B. dothidea* and *G. cingulata* were investigated (Table 1). Inhibition assay showed that the activity of *B. dothidea* PG I was reduced by 61.9%; from 0.957 of PG activity with apple PGIP to 0.365 of PG activity by crude extract of apple tissue (Table 1). Therefore, in all subsequent steps, we

Table 1. Inhibitory activities of apple polygalacturonase-inhibiting proteins (PGIPs) against polygalacturonase (PG) isozymes from *Botryosphaeria dothidea* and *Glomerella cingulata* in inoculated Fuji fruit.

Fungal pathogen	Isozyme	Specific activity of PG (units/mg of protein)	PG activity[a]		Inhibition (%)
			-PGIP	+PGIP[b]	
B. dothidea	PG	17.63	0.957	0.365	61.9
G. cingulata	PG	14.92	2.237	2.237	0
	PG	25.16	1.258	1.258	0

[a]Values are means of three assays.

[b]One unit of PGIP was added to each assay.

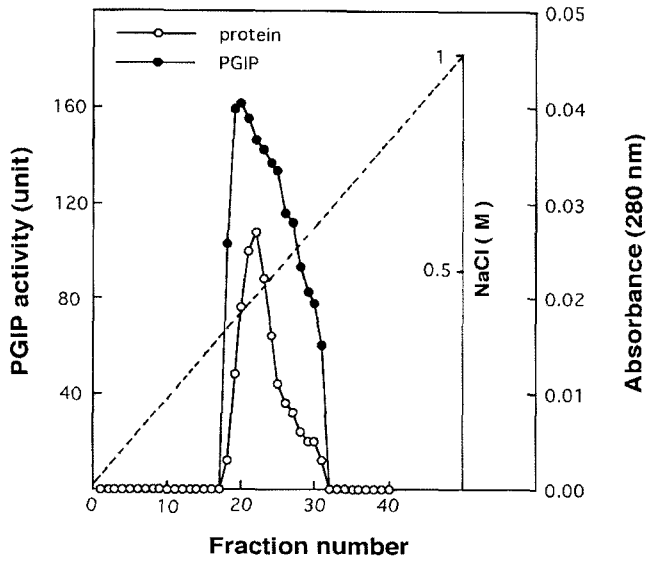

Fig. 1. Elution profile of polygalacturonase-inhibiting protein (PGIP) in QAE-sephadex A-50 ion-exchange chromatography.
Protein was eluted with a linear gradient (0–1 M NaCl in 10 mM Tris-HCl buffer; pH 8.0). Column size: 1.5×20 cm; Flow rate: 0.25 ml/min; Fraction volume: 2.5 ml.

examined the inhibitory activity of PGIP against PG. One peak of apple PGIP was observed in the fractions obtained from Sephadex G-100 gel filtration chromatography. This was further purified by chromatographies on CM-Sephadex and QAE-Sephadex ion-exchange columns (Figs. 1, 2). The active fractions were pooled, desalted, and concentrated

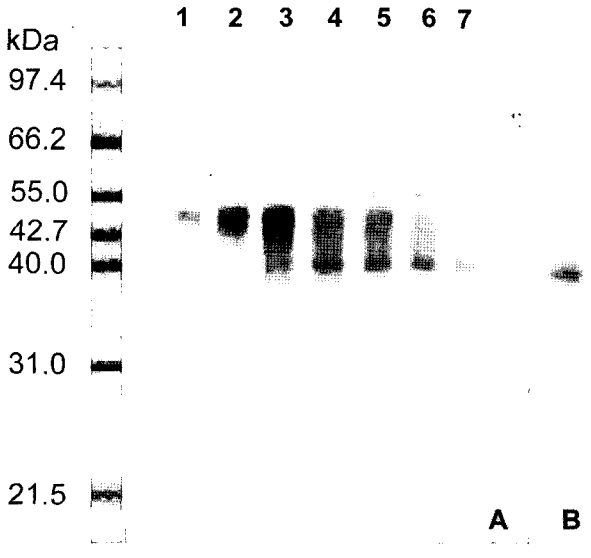

Fig. 2. SDS-PAGE of the PGIP in the fraction of purification.
Proteins were separated on 12.5% polyacrylamide gel and visualized by silver staining. **A.** PGIP fractions eluted from QAE-sephadex ion-exchange chromatography. **B.** PGIP eluted from Sephacryl S-200 gel filtration chromatography. The purified apple PGIP showed a molecular mass of 40 kDa.

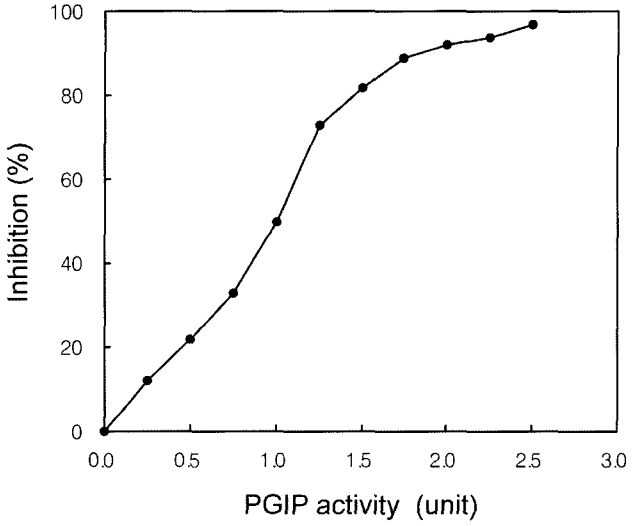

Fig. 3. Inhibition of PG purified from decayed tissue of *B. dothidea* in inoculated Fuji fruit by different amounts of purified apple PGIP.

by ultrafiltration. Two peaks of the PGIP activitiy after remained chromatographies on CM-Sephadex and QAE-Sephadex ion-exchange columns. Sephacryl S-200 chromatography was further used to remove glycosylated proteins. After the step to remove glycosylated proteins, only one protein band of approximately 40 kDa was detected by SDS-PAGE (Fig. 2).

PGIP showed differential inhibitory activity against PGs extracted from Fuji apple fruit inoculated with *B. dothidea* and *G. cingulata*. The purified apple PGIP inhibited the activity of PG from *B. dothidea* by 61.9% (Table 1). Further increase of the PGIP concentration resulted in progressive inhibition of PG activity from *B. dothidea* (Fig. 3). Complete inhibition of the *B. dothidea* PG activity required 2.5 units of PGIP. However, this inhibitory activity was not detected in assays performed with the two PGs from *G. cingulata* (Table 1).

Analysis of N-Terminal Amino Acid Sequences

The amino acid sequence of the N-terminus of purified Fuji apple PGIP was determined (Fig. 4). The N-terminal amino acid sequences of isolated Fuji apple PGIP shared homologies with other plant PGIPs. Sequence identities were 100% with Golden Delicious apple [38], 100% for

Fuji Apple	DLCNPDDKKVLLQIKKAFGDPYVLT
GD	DLCNPDDKKVLLQIKKAFGDPYVLT
Pear	DLCNPDDKKVLLQIKKAFGDPYVLA
Tomato	VRCNPKDKKVLLQIKKDLGNPYHLA
Bean	ELCNPQDKQALLQIKKDLGNPTTLS

Fig. 4. Comparison of N-terminal amino acid sequences of polygalacturonase-inhibiting protein (PGIP) of Fuji apple, Golden delicious apple (GD), pear, tomato, and bean.

Table 2. Contents of uronic acid in soluble materials extracted from apple fruit cell wall treated with *B.dothidea* PG alone or PG and PGIP together.

Treatment	Uronic acid content (μg/mg-cell wall)[a]
Untreated	106.8±0.2
PG[b]	118.7±0.3
PG+PGIP[c]	111.0±0.1

[a]Values are means of three assays.
[b]Fifty units of PG produced by *B. dothidea* was added to each assay.
[c]Forty units of PGIP was added to each assay.

pear [32], 70% for tomato [31], and 65% for bean [33], indicating that PGs of wood plants share high amino acid sequence homology.

Action of PG and PGIP on Soluble Cell Wall Material of Fuji Apple Fruit

When PG degrades plant cell walls by hydrolyzing pectin chains, short polymers of uronic acid and noncellulosic neutral sugars are produced. Therefore, we investigated the *in vivo* functions of *B. dothidea* PG and apple PGIP on the degradation of cell wall material extracted from mature Fuji apple fruit. This experiment would also demonstrate the biochemically relevant function of PGIP against the enzymatic active of PG. The results of the experiments are shown in Table 2. The uronic acid concentration of the untreated cell wall material was about 106.8 μg/mg, whereas it was increased to 118.7 μg/mg in the sample treated with 50 units of *B. dothidea* PG (Table 2). However, the uronic acid concentration was 111.0 μg/mg, when PG and PGIP were treated together. In the PG-treated cell wall sample, about 11.9 μg/mg of uronic acid was released, more than that of the untreated sample. However, in the sample that was treated with both PGIP and PG, only 4.2 μg/mg of uronic acid was released compared with that of the untreated sample. Therefore, according to the reduced release of uronic acid (Table 2), the inhibitory effect of PGIP against PG activity was about 65%. The amounts of noncellulosic neutral sugars were measured to study the PGIP inhibition activity against PG in each treatment (Table 3). Treatment of cell wall material with PG alone released large amounts of rhamnose, arabinose, and galactose from the main chain of rhamnogalacturonan. However, more amount of neutral sugars remained, when treated with both PGIP and PG than with PG alone (Table 3), indicating that the purified PGIP actively inhibits the enzymatic function of PG (Table 3).

Gel Chromatography of Pectic Cell Wall Fractions

To investigate the rate of the inhibitory activity of PGIP against PG, cell wall materials were subjected to size exclusion Sepharose CL-2B gel permeation chromatography. To estimate the molecular mass of the released uronic acid

Table 3. Neutral sugar contents in Fuji cell wall polymer fractions.

Treatment with	Rha[a]	Ara	Xyl	Man	Gal	Glc
	mg/10 mg					
Untreated	0.102[b]	0.536	0.471	0.083	0.390	0.233
PG[c]	0.088	0.418	0.441	0.069	0.332	0.224
PG+PGIP[d]	0.097	0.519	0.461	0.078	0.382	0.232

[a]Rha, Rhamnose; Ara, Arabinose; Xyl, Xylose; Man, Mannose; Gal, Galactose; Glc, Glucose.
[b]Values are means of triplicate analyses and for any value error was less than 10%.
[c]Fifty units of PG was added to each assay.
[d]Forty units of PGIP was added to each assay.

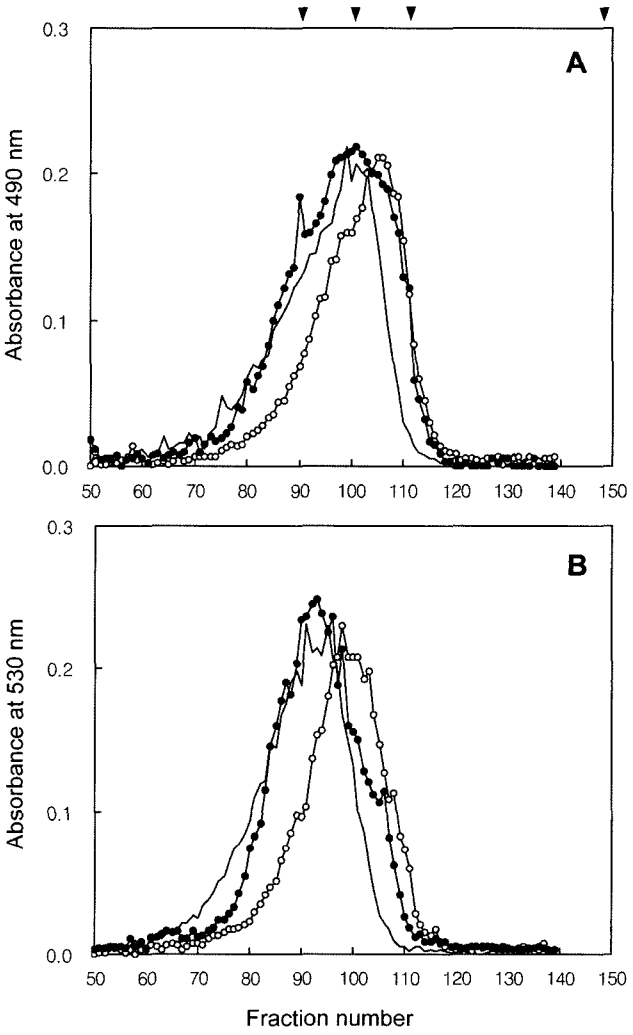

Fig. 5. Molecular mass changes of polygalacturonase-inhibiting protein (PGIP) treated cell wall material on Sepharose CL-2B.
Column fractions were assayed for total carbohydrate using the phenol-sulfuric acid method (**A**, 490 nm) and for uronic acid using the carbazole method (**B**, 530 nm). Arrows at the top of the figure represent elution positions (from left to right) of blue dextran with 2,000, 515, 73, and 42 kDa, respectively. (-○-): Fifty units of *B. dothidea* PG treated alone. (-●-): Fifty units PG from *B. dothidea* in inoculated Fuji fruit and forty units purified apple PGIP treatment. (—): Untreated.

polymers, we loaded the chromatography column with the blue dextran, and observed that dextrans were eluted corresponding to 2,000, 515, 73, and 42 kDa, respectively, from left to right (Fig. 5). When PG was treated alone, a large amount of high molecular weight uronic acid polymers (>2,000 kDa) were degraded, resulting in the reduction of large polymers (Figs. 5A and 5B). When PG and PGIP were treated together, less amount of high molecular weight uronic acid polymers were less degraded than PG treated alone (Figs. 5A and 5B).

Molecular mass shift was even more pronounced when noncellulosic neutral sugars were measured in polygalacturonase-inhibiting protein (PGIP)-treated water-soluble pectin extracted from cell wall material. These results are shown in Fig. 6. The water-soluble fraction was also subjected to gel permeation chromatography, and this fraction was also found to be composed of high molecular mass polymers in both total sugar (Fig. 6A) and uronic acid (Fig. 6B) in the treatment with both PG and PGIP together than that with PG alone (Fig. 6). The result also demonstrated that PGIP inhibited the activity of PG. Gel filtration of the CDTA fractions that had been treated with *B. dothidea* PG showed that PG was able to degrade pectic polymers (Fig. 7). PG was also able to degrade Na_2CO_3

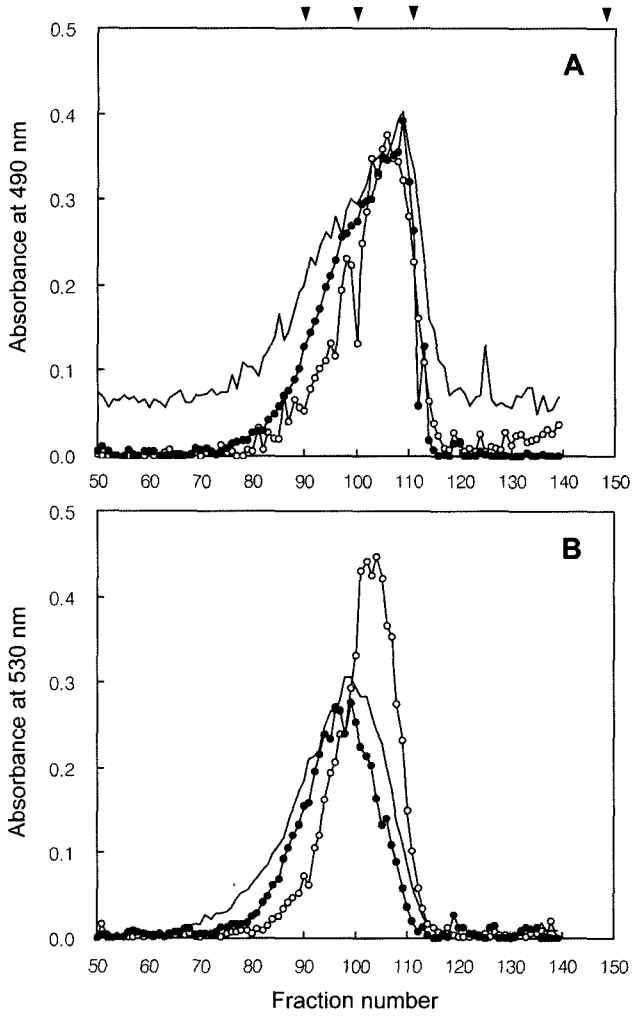

Fig. 6. Changes of molecular mass of polygalacturonase-inhibiting protein (PGIP) treated water-soluble pectin extracted from cell wall material on Sepharose CL-2B.

Column fractions were assayed using the phenol-sulfuric acid method (**A**, 490 nm) for total carbohydrate, and using the carbazole method (**B**, 530 nm) for uronic acid. Arrows at the top of the figure represent the elution positions from left to right of blue dextran with 2,000, 515, 73, and 42 kDa, respectively. (-○-): Fifty units PG from *B. dothidea* in inoculated Fuji fruit treatment. (-●-): Fifty units PG from *B. dothidea* in inoculated Fuji fruit and forty units purified apple PGIP treatment. (—): Untreated.

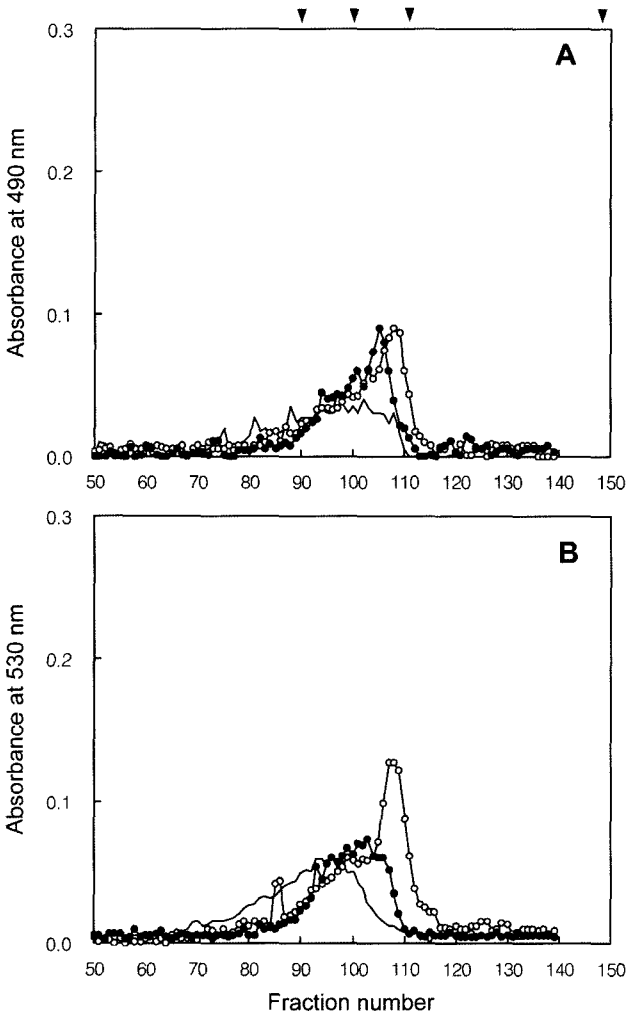

Fig. 7. Changes of molecular mass of polygalacturonase-inhibiting protein (PGIP) treated CDTA-soluble pectin extracted from cell wall material on Sepharose CL-2B.

Column fractions were assayed using the phenol-sulfuric acid method for total carbohydrate (**A**, 490 nm), and using the carbazole method (**B**, 530 nm) for uronic acid. Arrows at the top of the figure represent the elution positions from left to right of blue dextran with 2,000, 515, 73, and 42 kDa, respectively. (-○-): 50 units PG from *B. dothidea* in inoculated Fuji fruit treatment. (-●-): Fifty units PG from *B. dothidea* in inoculated Fuji fruit and forty units purified apple PGIP treatment. (—): Untreated.

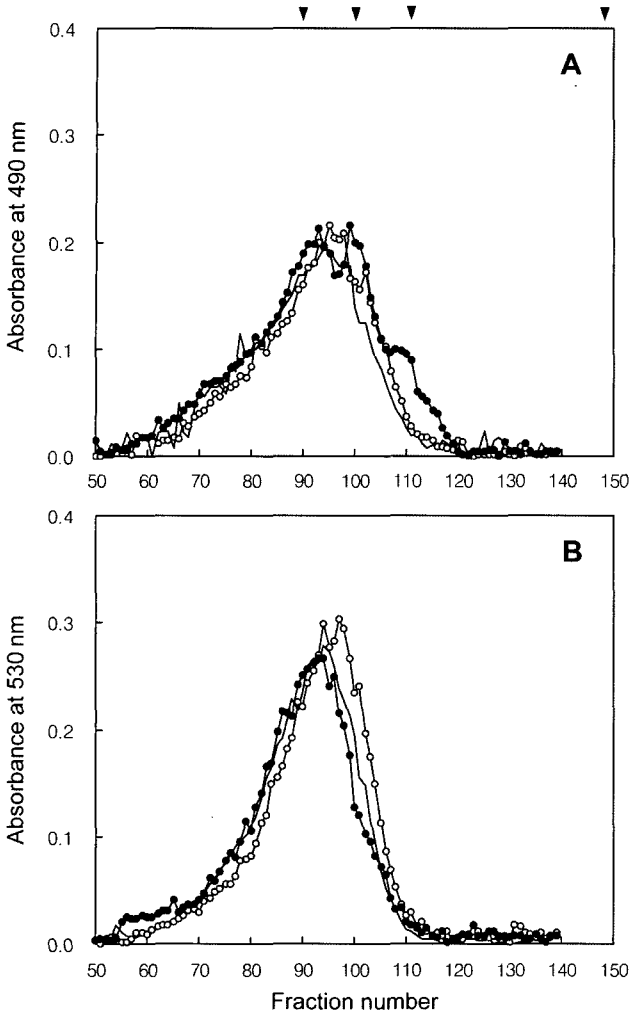

Fig. 8. Molecular mass changes of polygalacturonase-inhibiting protein (PGIP) treated Na_2CO_3-soluble pectin extracted from cell wall material on Sepharose CL-2B.

Column fractions were assayed using the phenol-sulfuric acid method for total carbohydrate (**A**, 490 nm), and using the carbazole method (**B**, 530 nm) for uronic acid. Arrows at the top of the figure represent the elution positions from left to right of blue dextran with 2,000, 515, 73, and 42 kDa, respectively. (-○-): Fifty units PG from *B. dothidea* in inoculated Fuji fruit treatment. (-●-): Fifty units PG from *B. dothidea* in inoculated Fuji fruit and forty units purified apple PGIP treatment. (—): Untreatec.

soluble pectin, whereas the soluble pectin was not degraded by the combined treatment of PGIP and PG, indicating the inhibitory activity of PGIP against PG (Fig. 8). This unmistakable degradation of pectin suggested that PG may be active in the apple cell wall.

DISCUSSION

PGIPs are extracellular plant proteins that are able to inhibit fungal PGs to protect plants from pathogens [11]. In this study, we isolated an apple PGIP and three PG isozymes from Fuji apple fruits that were inoculated individually with *B. dothidea* and *G. cingulata*, and examined both the catalytic properties of PGs and the inhibition of PGIP against PGs. Furthermore, we characterized the *in vivo* function of the PGIP on the solubilization and depolymerization of polyuronides from cell wall of apple fruits treated with PG.

The purified Fuji apple PGIP specifically inhibited the PG of only *B. dothidea*, but was less effective against the PGs of *G. cingulata* (Table 1). In fact, this specific inhibition of Fuji apple PGIP correlated to the fact that Fuji apple fruit was not sensitive to *G. cingulata*, but to *B. dothidea*. This observation is in accordance with previous studies, which showed that PGIPs from a single plant exhibit differential inhibitory activity against PGs from different fungi [1, 5, 18, 24, 25, 30, 38].

The N-terminal sequence of the isolated Fuji apple PGIP in Fig. 4 is the same as that of Golden Delicious apple [38] and they are highly conserved, indicating that the function of the proteins could be similar in not only apple cultivars but also other plants. However, the purified Fuji apple PGIP was approximately 40 kDa, which is the size similar to soybean PGIP [15], but differed from pear, tomato, and Golden Delicious apple PGIPs [31, 32, 38]. The PGIPs purified from Golden Delicious apple had a molecular mass of 44 to 50 kDa [38], suggesting that Fuji apple PGIP in this study could be different from Golden Delicious apple PGIP. The sequence of Fuji apple PGIP showed 100% identities with those of Golden Delicious apple [38] and pear [32], but 70% with tomato [31] and 65% with bean. This suggests that wood plants share a similar mechanism in the defense system using PGIP against pathogen.

We demonstrated that the PG isolated from *B. dothidea* successfully degraded cell wall components by measuring the release of uronic acid from pectic cell wall material. However, when PG was treated with apple PGIP, uronic acid contents were lower than those PG-treated alone. These results demonstrated that PG from *B. dothidea* cleaves the α-1,4 linkages between D-galacturonic acid residues in the rhamnogalacturonan, the main component of pectin, and separates cell walls from each other. These PG activities result in the maceration of plant tissue. When purified apple PGIP was treated with PG in cell wall material, cell wall degradation was significantly decreased, evidenced by measurement of the release of uronic acid and neutral total sugar, demonstrating that Fuji apple PGIP certainly has inhibitory activity against PG of *B. dothidea*.

In conclusion, we demonstrated that Fuji apple PGIP inhibited PG activity purified from decayed Fuji apple fruits after inoculation with *B. dothidea*. Furthermore, we also demonstrated that PGIP of a plant is one of the direct defense mechanisms against pathogen attack, by inhibiting PGs that are released from pathogens to hydrolyze the cell wall components of plants.

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