Purification and Characterization
of a Polygalacturonase-Inhibiting Protein from Apple Fruit

Chenglin Yao, William S. Conway, and Carl E. Sams

First and second authors: Horticultural Crops Quality Laboratory, USDA-ARS, BARC-West, Beltsville, MD 20705; third author: Department of Plant and Soil Science, University of Tennessee, Knoxville 37901.
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ABSTRACT

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A polygalacturonase-inhibiting protein (PGIP) was purified from mature 'Golden Delicious' apple fruit. The protein was cell wall bound and had a molecular mass of 44 to 54 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Chemical deglycosylation of purified apple PGIP released a 34-kDa polypeptide, suggesting that differential glycosylation accounted for the heterogeneity in molecular mass. Apple PGIP showed differential inhibitory activity against five polygalacturonase isozymes purified from Botrytis cinerea grown in liquid culture. However, inhibition was not detected against polygalacturonase extracted from apple fruit inoculated with the same fungus. Kinetic studies suggested a mixed-type inhibition. N-terminal amino acid sequence of apple PGIP shared 96%, 68%, and 60% identity with those from pear, tomato, and bean, respectively.

Plant pathogens produce a number of cell wall-degrading enzymes (12,14). Polygalacturonase (PG) (EC 3.2.1.15) is the first enzyme secreted by plant fungal pathogens when cultured on isolated cell walls (23). Degradation of plant cell walls by PG facilitates the attack of other cell wall-degrading enzymes on their substrates (25). A role for PG in pathogenicity has been proposed for soft-rot pathogens because they cause extensive degradation of plant cell walls leading to the maceration of host tissue (5,12,15).

Plant cell walls contain proteins that can specifically and effectively inhibit PGs of fungal origin. These polygalacturonase-inhibiting proteins (PGIP) have been reported from numerous plant species (1,2,8,9,10,11,16,17,18,21,22,32), but only a few of those proteins have been purified to homogeneity (11,22,32,33). Biochemical characterization of PGIPs shows that they are glycoproteins (32,33) and relatively heat stable (1,2,8). Some PGIPs display heterogeneity in molecular mass caused by differential glycosylation of a single polypeptide (32,33). Kinetic studies of PGIPs revealed that some inhibit fungal PGs by a competitive-type mechanism (1), whereas others are noncompetitive (22,27). Furthermore, the inhibition of PG by PGIPs is highly specific. PGIP from a single plant source can differentially inhibit PGs from several fungal species (1,2,8,10,19,22) or PG isozymes from one fungus (22,31). PGIPs from different plants inhibit PG from a single fungal species to different extents (32). For example, pear PGIP inhibits PG from culture filtrates of Botrytis cinerea Pers.:Fr. more strongly than does PGIP from tomato. PGIP has been shown to be a disease resistance factor against pathogen infection. Ripening tomato fruit from transgenic plants expressing the pear PGIP gene are more resistant to B. cinerea infection than the control fruit (29).

The loss of apple fruit in storage is substantial due to decay caused by postharvest pathogens such as B. cinerea and Penicillium expansum. Although fungicides can effectively control some of these pathogens, public concerns about health and environmental impact limit their future application. Since PGIP has been proven to be a plant defense mechanism against pathogen infection, it may be suitable as an alternate method to control postharvest diseases. Proteinaceous inhibitors of fungal PGs have been detected in apple leaves (28) and in infected and healthy apple fruit (8,18). However, apple PGIP has not been purified to homogeneity or characterized. This paper describes, for the first time, the purification and characterization of a PGIP from mature 'Golden Delicious' fruit (Malus domestica Borkh.) and its activity against different PG isozymes produced by B. cinerea in liquid culture and inoculated fruit.

MATERIALS AND METHODS

Fungal and plant materials. B. cinerea was isolated from naturally infected apple fruit and maintained on potato-dextrose agar (deposited as ATCC 90870 at the American Type Culture Collection, Rockville, MD). 'Golden Delicious' apple fruit were harvested from a commercial orchard in Pennsylvania.

Preparation of fungal PGs. To produce PGs in liquid culture, 10 mycelial plugs (0.5 cm²) from B. cinerea cultures were used to inoculate 1 liter of modified Richard's solution (34). The culture was incubated at 20°C with continuous shaking (100 rpm) for 10 days. The culture filtrate was concentrated to 10 ml with minitan (Millipore Corp., Bedford, MA) and stirred cell (Amicon, Beverly, MA) ultrafiltration systems equipped with 10-kDa cut-off low protein-binding membranes. The concentrate was dialyzed against 25 mM Bis-Tris-HCl (pH 6.0) overnight and loaded onto a chromatofocusing column (Mono P HR 5/20; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The five PG isozymes were separated according to the protocol of Tobias et al. (34). To isolate PG produced by B. cinerea in apple cortical tissue, 'Golden Delicious' fruit were inoculated with a spore suspension (10^5 conidia/ml) as previously described (13,35). After 8 days of incubation at 20°C, rotted tissue (500 g) was collected and used for

PG purification (35). Some characteristics of these PGs are listed in Table 1.

Extraction and purification of apple PGIP. After storage at 4°C for 90 days, 5 kg of apple cortical tissue was used for PGIP extraction. Apple tissue (in 500-g portions) was homogenized in equal volumes of 50 mM sodium acetate, 1.5% polyvinyl-polypyrrolidone (PVPP), and 0.2% sodium bisulfate (pH 6.0). After filtration through Miracloth (Calbiochem-Behring, La Jolla,

TABLE 1. Some characteristics of polygalacturonase (PG) isozymes produced by *Botrytis cinerea* in liquid culture and inoculated fruit

Source	Isozyme	Mode[a]	Specific activity (units/mg of protein)
	PG I	endo-	10.3
	PG II	exo-	40.1
In culture	PG III	exo-	59.6
	PG IV	exo-	8.7
	PG V	exo-	7.2
In fruit	PG	exo-	109.5

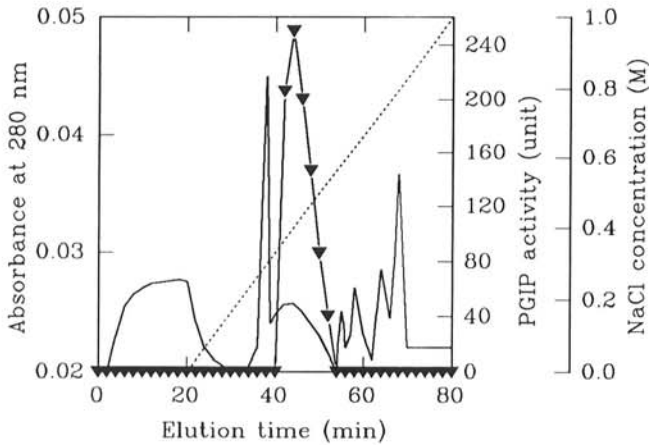[a] Cited from references 34 and 35.

Fig. 1. Elution profile of apple extract on Mono S column. Polygalacturonase-inhibiting protein (PGIP) was eluted using a 30-ml linear 0 to 1 M sodium chloride gradient in 50 mM sodium formate (pH 4.0). Absorbance of fractions at 280 nm (–); PGIP activity (▼); and salt gradient (…).
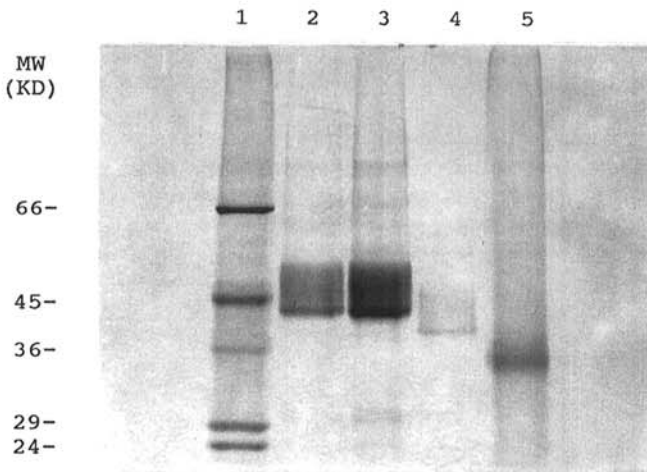

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of apple polygalacturonase-inhibiting protein (PGIP) after enzymatic and chemical deglycosylation. Lane 1: molecular weight markers; lane 2: nontreated; lane 3: O-glycosidase-treated; lane 4: N-glycosidase-treated; lane 5: triflouromethane sulfonic acid-treated. Note: the different amounts of protein in each lane were due to the variation of recovery in the precipitation procedure.

CA), insoluble tissue was washed three times by brief homogenization in 500 ml of 50 mM sodium acetate and 0.2% sodium bisulfate (pH 6.0). The homogenate was filtered through Miracloth each time. The remaining insoluble tissue was resuspended in 1 liter of 50 mM sodium acetate, 1 M sodium chloride, and 0.2% sodium bisulfate (pH 6.0), and stirred for 30 min at 4°C. Following filtration, the remaining tissue was reextracted. The sodium chloride extracts were combined and centrifuged at 17,700 × g for 30 min. The supernatant was concentrated to 100 ml by a minitan ultrafiltration system equipped with 10-kDa cut-off low protein-binding membranes. The concentrate was divided into 10-ml aliquots and stored at –20°C.

Apple PGIP was purified by fast protein liquid chromatography (FPLC) (Waters Chromatography Div., Milford, MA) using a Mono S HR 5/5 cation exchange column (Pharmacia LKB Biotechnology Inc.). After dialysis against 50 mM sodium formate (pH 4.0) (buffer A) overnight, the sample was applied to the column equilibrated with buffer A. Proteins were eluted at 0.5 ml/min with a 30-ml linear gradient from 0 to 1 M sodium chloride in buffer A. Fractions (1 ml) were collected and used to assay PGIP activity.

PGIP activity assay. The inhibition of PG activity was determined by measuring reducing groups released from sodium polypectate in the absence and presence of PGIP, using D-galacturonic acid as the standard (20,34). One unit of PG activity was defined as the amount of enzyme required to release 1 μmol of reducing groups per minute at 37°C in a mixture containing 0.176% sodium polypectate and 80 mM sodium acetate (pH 5.0). The same mixture was used to assay PGIP activity. One unit of PGIP was defined as the amount of inhibitor required to reduce the activity of 0.05 unit of *B. cinerea* PG II by 50%.

Preparative isoelectric focusing (IEF). This method was used to estimate the pI of the inhibitor. One aliquot of concentrated sample was extensively dialyzed against deionized water for 24 h, and subjected to IEF using a Rotofor cell (Bio-Rad, Hercules, CA) in the 3 to 10 pH range.

Protein assay and gel electrophoresis. Protein was determined by the method of Bradford (7) using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in a Bio-Rad Mini-protean II cell according to Laemmli (26). The gel was stained with a Bio-Rad silver stain kit according to the recommendation of the manufacturer. The molecular mass of the proteins was estimated by comparison to Sigma SDS-7 molecular weight markers (Sigma Chemical Co., St. Louis).

Deglycosylation of PGIP. Aliquots of samples containing 1 μg of purified inhibitor were used for enzymatic deglycosylation analyses. To remove N-linked glycans, aliquots were denatured by boiling for 5 min in 1% sodium dodecyl sulfate (SDS), and then incubated with 1 unit of N-glycosidase F (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37°C for 18 h in 100 μl of reaction mixture containing 20 mM sodium phosphate (pH 8.0), 10 mM EDTA, 0.2% SDS, 1% Triton X-100, and 1% 2-mercaptoethanol. Nondenatured aliquots were also deglycosylated by O-glycosidase (Boehringer Mannheim Biochemicals) at 37°C for 18 h in 100 μl of 20 mM sodium phosphate buffer (pH 6.0) in the presence of 1 milliunit of enzyme. Following incubation, the reaction mixture was adjusted to contain 125 μg/ml of sodium deoxycholate (6) and kept at room temperature for 15 min. Protein was then precipitated by 6% trichloroacetic acid (TCA) and used for SDS-PAGE.

Purified PGIP was chemically deglycosylated with tri-flouromethane sulfonic acid (TFMS; Fluka Chemical Corp., Ronkonkoma, NY). After lyophilizing overnight in a 100-μl Reacti-Vial (Pierce Chemical Co., Rockford, IL), the sample containing 10 μg of protein was treated according to the protocol developed by Karp et al. (24) with some modifications. After

dialysis against 0.1 M ammonium bicarbonate overnight, the sample was recovered by 6% TCA precipitation in the presence of sodium deoxycholate as described above.

N-terminal sequencing. The purified inhibitor (10 µg) was used to run a SDS-PAGE and electroblotted onto a polyvinylidene diflouride (PVDF) membrane (Bio-Rad) according to the method described by Towbin et al. (37), and stained with 0.5% Ponceau S in 0.1% acetic acid (30). The N-terminal amino acid sequence of PGIP was determined at the Harvard Microchemistry Facility (Cambridge, MA) using an ABI 494 protein sequencer equipped with an 190 PTH analyzer.

RESULTS

Extraction and purification of PGIP. The sodium chloride extract of apple cortical tissue contained approximately 70% inhibitory activity against PG II isolated from *B. cinerea* grown in liquid culture, which indicated that PGIP is cell wall bound. Since most PGIP activity was present in the sodium chloride extract, it was selected as the source for PGIP purification. Following extensive dialysis against deionized water, the extract was used for isoelectric focusing in the 3 to 10 pH range. The fraction showing the highest inhibitory activity had a pH of 4.6. However, PGIP was not electrofocused effectively, because the activity was detected in nine fractions with a pH ranging from 3.0 to 5.9. Based on the information that apple PGIP has an acidic pI, an initial attempt was made to purify the inhibitor using a Mono Q HR 5/5 anion exchange column (Pharmacia LKB Biotechnology Inc.) equilibrated with 20 mM Bis-Tris-HCl (pH 6.5). Proteins were eluted with a linear gradient from 0 to 1 M sodium chloride in 20 mM Bis-Tris-HCl (pH 6.5). Recovery of inhibitory activity was minimal, suggesting that PGIP binds very tightly to the anion exchange column. When the sample was applied onto a Mono S cation exchange column equilibrated with 50 mM sodium formate (pH 4.0), PGIP was eluted in 0.4 M sodium chloride (Fig. 1). When the buffer pH was raised from 4.0 to 4.3, most of the PGIP could not bind to the Mono S, which further confirmed that the inhibitor has an acidic pI. When the combined active fractions from the Mono S column were subjected to SDS-PAGE, a diffuse band with a molecular mass ranging from 44 to 54 kDa was detected (Fig. 2). The five individual fractions with inhibitory activity from the Mono S column were also analyzed by SDS-PAGE. The results indicated that PGIP from each fraction had a different range of molecular masses, with the largest molecule (54 kDa) eluted first and the smallest (44 kDa) eluted last (data not shown). Approximately 45% inhibitory activity in the minitan concentrate was recovered after chromatography on a Mono S column, with a 37-fold increase in specific activity (Table 2).

Deglycosylation of apple PGIP. Purified apple PGIP was treated with *N*-glycosidase F, *O*-glycosidase, and TFMS, respectively. After incubation with *O*-glycosidase, no change in mobility on the gel was observed (Fig. 2). Incubation with *N*-glycosidase F resulted in an increase in mobility of the diffuse band. Furthermore, chemical deglycosylation revealed a single polypeptide with a molecular mass of 34 kDa (Fig. 2). This indicated that apple PGIP is a glycoprotein, and the heterogeneity in molecular mass is caused by differential glycosylation of a single polypeptide. There was no detectable amount of O-linked oligosaccharides associated with the inhibitor, and some of the N-linked glycan was not accessible to *N*-glycosidase F.

Inhibition of PGs by PGIP. When six PG isozymes produced by *B. cinerea* were incubated with the same amount of inhibitor, differential inhibition was observed. Four PGs (PG I to PG IV) isolated from liquid culture were significantly inhibited by apple PGIP, but little inhibition was detected for PG V. No inhibition was observed for the PG purified from apple fruit inoculated with *B. cinerea* (Table 3). When different units of the inhibitor were assayed against PG II, it was found that the degree of inhibition

was dependent on the amount of PGIP present in the reaction mixture (Fig. 3).

Mechanism of inhibition. PG II activity was determined in the absence and presence of PGIP over a range of polygalacturonic acid (0.011 to 0.044%). A double-reciprocal plot of enzyme kinetics revealed that both V_{max} and K_m were affected by the inhibitor (Fig. 4), which indicated that apple PGIP is a mixed-type inhibitor against PG II from *B. cinerea*.

Heat stability of PGIP. Aliquots of purified inhibitor were boiled for 10, 20, and 30 min. When assayed with PG II, it was found that only 23, 16, and 12% activity remained, respectively.

N-terminal sequence analysis. Twenty-five amino acids were determined from the N-terminus of apple PGIP. When compared to protein sequences in the GenBank, significant homologies were observed between apple PGIP and three previously characterized PGIPs. The N-terminal amino acid sequence of apple PGIP showed 96%, 68%, and 60% residual identities with those of pear, tomato, and bean PGIPs, respectively (32,33,36). Three stretches of amino acid sequences (-CNP-, -DK-, and -LLQIKK-) were conserved among all four PGIPs (Fig. 5).

TABLE 2. Extraction and purification of polygalacturonase-inhibiting protein (PGIP) from 'Golden Delicious' apple cortical tissue

	Total activity (unit)	Total protein (mg)	Specific activity (units/mg of protein)
NaCl extract	n.d.[a]	36.78	...
Minitan concentrate	20,400	18.14	1,124
Mono S	9,280[b]	0.223[b]	41,614

[a] The amount of PGIP was too diluted to be measured directly.
[b] Values are estimates based on data obtained in a single chromatographic run when 1.814 mg of protein was loaded on the Mono S column.

TABLE 3. Differential inhibition of apple polygalacturonase-inhibiting protein (PGIP) of polygalacturonase (PG) isozymes from *Botrytis cinerea*

		PG activity[a]		
Source	Isozyme	−PGIP	+PGIP[b]	Inhibition (%)
In culture	PG I	0.052	0.017	67
	PG II	0.056	0.022	61
	PG III	0.043	0.034	21
	PG IV	0.048	0.043	10
	PG V	0.047	0.046	2
In fruit	PG	0.058	0.058	0

[a] Values are means of two assays.
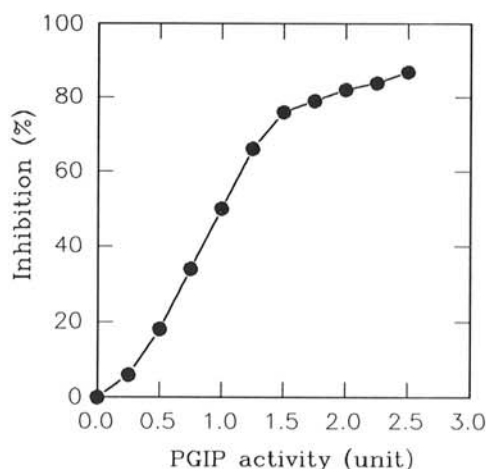[b] 1.2 units of PGIP were added to each assay.

Fig. 3. Inhibition of polygalacturonase (PG) II from *Botrytis cinerea* grown in liquid culture by different amounts of purified apple polygalacturonase-inhibiting protein (PGIP).

DISCUSSION

This report is the first time apple PGIP has been purified to homogeneity, the N-terminal amino acid sequence determined, and the inhibitory activity tested against purified PG isozymes produced by *B. cinerea*. Confirmation that apple PGIP was purified to homogeneity is supported by the data from both deglycosylation and N-terminal amino acid sequence analysis. Biochemical characterization of purified apple PGIP suggested that the heterogeneity in molecular mass is caused by posttranslational modifications of the same polypeptide with different glycans, as reported for PGIPs from tomato and soybean (17,32). The inhibitor was very similar to pear PGIP at the N-terminus. Among the 25 amino acids determined, 24 were identical. Significant similarities were also observed with tomato and bean PGIPs. After complete deglycosylation, the molecular mass of the apple PGIP (34 kDa) was the same as determined for pear and tomato PGIPs (32,33). The most notable difference between apple PGIP and other characterized PGIPs was the pI of approximately 4.6, as estimated by preparative IEF and binding properties to the Mono S column. In contrast, other PGIPs have basic pIs, the exception being pear PGIP which has isoforms with pIs of 4.5, 6.6, and 7.7, respectively (1). The apple inhibitor also had charge heterogeneity, as implied by the fact that it could not be efficiently electrofocused. This charge heterogeneity was probably due to the nonuniform glycosylation of the PGIP polypeptide. Apple PGIP showed mixed-type inhibition, as opposed to the competitive inhibition of pear PGIP (1) and noncompetitive inhibition of PGIPs from bean and raspberry (22,27).

PGIP from apple leaves has a pI of 9.3 and a molecular mass of 36 kDa (28). This protein showed differential inhibition against PGs from *Venturia inaequalis* and *Aspergillus niger*. However, a positive correlation between resistance of apple leaves to *V. inaequalis* and the PGIP content of leaves was not established. PG
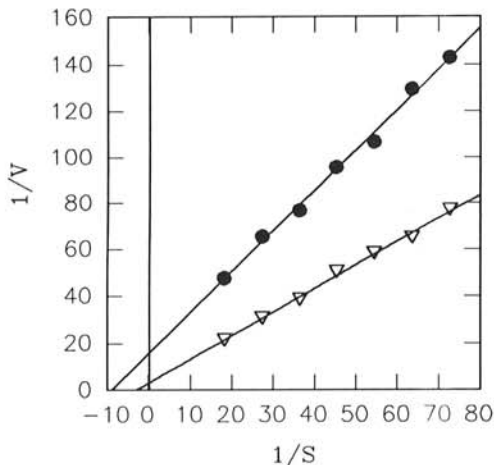
inhibitors with different pIs were present in apple fruit when inoculated with *Monilinia fructigena*, *M. laxa* f. sp. *mali*, *Nectria galigena*, and *B. cinerea*, but not with *Penicillium* sp.-infected fruit or healthy fruit tissue (18). This suggests that the formation of these inhibitors was initiated by the infection process.

An inhibitor from the cell wall of apple fruit also has been isolated and partially purified (8). This inhibitor had the ability to suppress the activity of endo-PGs produced in fruit and in culture by *N. galligena*, *Phomopsis mali*, *Fusarium lateritium*, and *Glomerella cingulata*, but had no effect on the endo-PGs produced by *Penicillium expansum* or *Phytophthora syringae*. Decay development caused by *N. galligena* was inversely related to inhibitor activity in the fruit tissue. The properties of this partially purified inhibitor from apple fruit are similar to the one we purified (e.g., its proteinaceous nature, heat stability, and the presence of carbohydrate).

B. cinerea produced five PG isozymes when grown in liquid culture using apple pectin as the sole carbon source. However, only one PG was isolated from apple fruit inoculated with the same fungus (34,35). Some similarities were observed between PG produced by *B. cinerea* in the fruit and PG III isolated from the culture, such as pI, optimal pH, and mode of action when hydrolyzing polypectate (35). These similarities suggest that the PG isozyme from the decayed apple tissue may be of fungal origin. One exo-PG has been detected in healthy 'Cox's Orange Rippin' apple fruit (4). However, its molecular mass and pH optima differ from the PG isozyme produced in decayed apple tissue (35). Endo-PG was reported to be present in 'McIntosh' apple fruit (38), but the amount of flesh needed to extract this enzyme was many times greater than that needed to extract the PG isozyme produced in the apple tissue decayed by *B. cinerea*. This enzyme is an endo-PG, as was the enzyme recently reported to be present in healthy 'Golden Delicious' apples (3). Despite using procedures and amounts of healthy tissue from 'Golden Delicious' fruit similar to that for which we extracted a PG isozyme from decayed fruit tissue, we were unable to detect any PG activity in healthy fruit.

The absence of multiple PG isozymes in inoculated apple fruit may suggest that PGIP is involved in limiting the production of most PG isozymes during fungal infection of fruit. This means that apple PGIP contributes to the general resistance mechanisms of fruit when fighting fungal infection. Although the inhibitor did not show inhibitory activity against the PG isozyme isolated from inoculated fruit when assayed in vitro, this does not exclude the possibility that apple PGIP still contributes to the overall resistant performance of plant. In fact, transgenic tomato fruits expressing the pear PGIP gene showed more resistant to *B. cinerea* than the control fruits (29). By keeping the PGIP gene constitutively expressed in apple fruit, or retarding the dissociation of PGIP from the cell wall using calcium and heat treatments, it is feasible to reduce decay caused by postharvest pathogens.

Fig. 4. Lineweaver-Burk double-reciprocal plot of polygalacturonase (PG) II activity in the absence (∇) and presence ($\bullet$) of 1.2 units of purified polygalacturonase-inhibiting protein (PGIP). V: PG activity in units; S: substrate concentration (wt/vol).

apple	DL	CNP	D	DK	KV	LLQIKK	AF	G	D	P	YV	L	T
pear	DL	CNP	D	DK	KV	LLQIKK	AF	G	D	P	YV	L	A
tomato	VR	CNP	K	DK	KV	LLQIKK	DL	G	N	P	YH	L	A
bean	EL	CNP	Q	DK	QA	LLQIKK	DL	G	N	P	TT	L	S

Fig. 5. Comparison of the N-terminal amino acid sequences of polygalacturonase-inhibiting proteins (PGIP) from apple, pear (33), tomato (32), and bean (36). The identical amino acids in all sequences are boxed.

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