The Purification, Physical/Chemical Characterization, and cDNA Sequence of Cantaloupe Fruit Polygalacturonase-Inhibiting Protein

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ABSTRACT

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Polygalacturonase-inhibiting proteins (PGIPs) are believed to aid in plant defense against fungal pathogens by inhibiting polygalacturonases (PGs) secreted by the invading organism. In an effort to better understand this type of plant-pathogen interaction in cucurbits, we have isolated a cantaloupe PGIP (CmPGIP) from 5 to 15 day postanthesis cantaloupe fruit. CmPGIP inhibited crude extracts of PG from two of four fungal pathogens of cantaloupe that were tested. Results from assays for PG activity that utilized rate of substrate viscosity reduction or rate of reducing group formation were consistent with CmPGIP inhibition of endo-PG

activity. The M_r of CmPGIP by sedimentation equilibrium or MALDI-TOF MS was 38,500. The pI of CmPGIP was ≈ 8.2 , and its absorptivity at 280 nm was 0.93 ml/mg. The circular dichroism spectrum of native CmPGIP exhibited strong negative ellipticity in the near UV and possessed a far UV spectrum indicative of β -sheet periodic structure. Amino acid sequences of the N terminus and a cyanogen bromide peptide were used to construct oligonucleotide primers for polymerase chain reaction sequencing. The sequenced open reading frame predicts a mature protein of 307 amino acids with up to 68% identity to other PGIP molecules. Northern blot analysis revealed differential expression during fruit development. The isolation and structural information obtained for CmPGIP by this investigation provide a foundation for the development of molecular strategies for pre- and postharvest crop protection.

Plants are continually exposed to a broad spectrum of fungal pathogens, and one of the barriers that plants employ against the onslaught is their cell wall. The fungal pathogen breaches this multipolysaccharide barrier and gains access to the plant tissue by secreting a number of enzymes capable of partially degrading the structural cell wall polymers. Because pectin is a major component of plant cell walls, one of the classes of hydrolytic enzymes frequently put forth as important, if not critical, to fungal pathogenesis is endopolygalacturonases (PGs). They are among the first enzymes secreted when fungi are grown on plant cell walls in vitro (24,30,37), and their action appears to facilitate other cell wall degradative enzymes reaching their respective substrates (7,25). In the early 1970s, glycoproteins were extracted from bean hypocotyl tissues, tomato stems, and sycamore cells that inhibited selected fungal PGs in vitro (3). Similar proteins from other plant families were subsequently isolated, and the designation, polygalacturonase inhibiting protein, or PGIP, was introduced.

The strength and specificity of the interaction between plant PGIPs and fungal PGs vary widely (reviewed in literature citation 13). PGIPs from a single plant tissue will differentially inhibit PGs from various fungi (1,11,15) or iso-PGIPs from the same fungus (43). The complex that is formed between the enzyme and inhibitor in vitro results in an alteration in the balance of products

derived from the action of PG on pectin (22). PG-PGIP complex formation favors a release of elicitor-active oligogalacturonides over the customary PG-catalyzed hydrolysis of pectin to di- and trigalacturonides. *pgip* genes have now been cloned from a wide array of plant species where they usually exist as gene families (13). Analyses of gene sequences have indicated that PGIPs possess the versatile structural motif of leucine-rich repeats (LRRs) (12,39) found in many proteins and appear to be involved in protein-protein interactions (23).

Cucurbits, which are an important food source worldwide, are subject to a broad spectrum of fungal diseases. In light of their economic importance internationally, we found it curious that no information has been forthcoming about cucurbit-derived PGIPs. Literature is limited to two reports that substantiate their presence (27,37). The purpose of this research was to isolate and characterize PGIP from a member of the cucurbit family as a prelude toward developing an understanding of its interactions with PGs from a selection of cucurbit fungal pathogens.

MATERIALS AND METHODS

Reagents. Oligonucleotide primers were synthesized by Gibco BRL Invitrogen Life Technologies (Gaithersburg, MD). Total RNA was isolated using TRIzol reagent (Gibco BRL Invitrogen Life Technologies). BrightStar Biotinylated RNA Millennium RNA Markers (Ambion, Austin, TX) were used for size standards on denaturing RNA gels, which were run using the NorthernMax Kit (Ambion). Visualization of *pgip* mRNA was performed using BrightStar Detection Kits (Ambion) and a Kodak Image Station 440CF (Eastman Kodak Co., Rochester, NY).

Source materials for PG. Fungal pathogens, *Phomopsis cucurbitae*, *Fusarium solani*, *F. verticillioides* (synonym = *F. moniliforme*; J. Sheld; teleomorph = *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura, mating population A), and *Didymella bryoniae* were provided by B. Bruton, U.S. Department of Agriculture. Fungal PG was prepared from cantaloupe fruit by inocu-

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lating cantaloupe fruit according to published procedures (44–46). Mature, full-slip fruit were inoculated with *F. solani*, *F. verticillioides*, or *P. cucurbitae*, whereas 10-day-old fruit were used to grow *D. bryoniae*. Lesions were harvested 6 days after inoculation for all organisms except those of *F. verticillioides* that were 8 days old. An enzyme extract was prepared from lesions by grinding in an equal weight of 2 M NaCl in 0.05 M sodium acetate buffer, pH 5.0. After centrifugation at $10,000 \times g$ at 15°C for 30 min to remove insoluble material, the extract was concentrated ≈ 20 - to 50-fold on an Amicon YM-10 membrane (Millipore Corp., Bedford, MA) and transferred to 0.05 M sodium acetate buffer, pH 5.0, by diafiltration with the Amicon YM-10 membrane. Commercial preparations of PG from *Aspergillus niger* and *Rhizopus* sp. (Sigma Chemical, St. Louis) were used without further purification.

Purification of cantaloupe PGIP. ‘Copa de Oro’ cantaloupe (*Cucumis melo* var. *reticulatus*) from the 2000 crop at Lane Ag Center (Lane, OK) were harvested at 5 to 10 days postanthesis. Fruit were diced, and 3.6 kg of tissue was added to 400 ml of deionized water and blended for 5 min (Waring Products, New Hartford, CT). The ground material was frozen in zip-lock plastic bags at -15°C until processing. The ground cantaloupe tissue was thawed and preextracted in batches of 1.8 kg (1.6 kg of tissue). The preextraction process consisted of washing the tissue solids five times with 2 liters of water, once with 2 liters of 0.1 M NaCl in 0.05 M sodium phosphate buffer, pH 7.2, and three times with 0.05 M sodium phosphate buffer, pH 7.2. Solubilization of PGIP from the prewashed cantaloupe tissue was accomplished by extraction with 2 liters of 0.6 M NaCl in 0.05 M sodium phosphate buffer, pH 7.2, by stirring at 7°C for 2 h. This was followed by separation of liquid and solids by filtration through parachute cloth and centrifugation at $10,000 \times g$ for 30 min at 15°C . The material that solubilized in the 0.6 M NaCl was concentrated from 2 liters to ≈ 150 ml on a Minitan membrane concentrator (Millipore) with a Minitan Biomax PB plate of 10,000 MW cut-off. The extract (≈ 150 ml) was further concentrated to ≈ 15 ml with a YM-10 membrane (Millipore) in an Amicon concentrator. The crude, concentrated cantaloupe PGIP (CmPGIP) extract was diafiltered into 0.05 M sodium phosphate buffer, pH 7.2, while in the Amicon concentrator.

The CmPGIP extract was subjected to cation exchange chromatography on a strong cation exchanger (Bio-Rad MP25S; Bio-Rad Laboratories, Hercules, CA) packed in a bed (12 by 85 mm) in a column (Bio-Rad MT10). Gradient elution and effluent monitoring at 280 nm were performed with an advanced protein purification system (Waters 650E; Waters Corp., Milford, MA) and a tunable absorbance detector (Waters 484). The column was operated at a flow rate of 1.5 ml/min. The elution program after injection of the sample in 0.05 M sodium phosphate buffer, pH 7.2, was as follows: (i) 10 min isocratic elution with 0.05 M sodium phosphate buffer, pH 7.2, (ii) a linear gradient from buffer only to buffer plus 0.5 M NaCl over 60 min, (iii) a linear gradient from 0.5 M NaCl to 1 M NaCl in buffer over 10 min, (iv) isocratic elution at 1 M NaCl in buffer for 10 min, and (v) a 5 min linear gradient return to buffer only followed by 15 min of column reequilibration in buffer.

Gel filtration was performed on a bed (1.6 by 92.5 cm) of BioGel P-100 (Bio-Rad) in a Pharmacia column (Amersham Pharmacia Biotech, Piscataway, NJ). The gel filtration medium was equilibrated in 0.2 M sodium acetate buffer, pH 5.0. The flow rate, as maintained by a Pharmacia peristaltic pump, was 4 ml/h (2 cm/h linear flow rate). One-milliliter samples were placed on the P-100 column and eluted at the above flow rate. Fractions (≈ 2 ml) with constant specific activity for PGIP were pooled and concentrated in a Centriprep YM-10 (Millipore) tube to 5 ml.

PG and CmPGIP activity assays. PG activity was routinely assayed by measuring reducing groups released from sodium polygalacturonate by the method of Gross (20). D-Galacturonic

acid was used as the standard to convert absorbance change to molar concentration of reducing groups formed. Assays were conducted at $30 \pm 1^{\circ}\text{C}$ in 0.05 M sodium acetate, pH 5.0, at a substrate concentration of 0.1% (wt/vol) polygalacturonate. Apparent initial rates were estimated by a discontinuous assay where duplicate samples were removed from the incubation mixture at 0, 2.5, 5, and 7.5 min. The square of the correlation coefficient, R^2 , for a linear least-squares fit to the eight points in an assay routinely ran 0.99 ± 0.01 . Inhibition of PG activity by CmPGIP was estimated by conducting the assay for PG in the absence and presence of CmPGIP. Five to seven nanokatals (1 nmol of reducing groups formed per second) of PG activity were employed in each assay. PG and CmPGIP were preincubated at room temperature for 15 min followed by incubation at 30°C for 5 min before assaying residual PG activity. Following precedence established in earlier studies (3,8,40,43), one unit of PGIP was defined as the amount of inhibitor required to reduce one unit of PG activity by 50%.

In some instances, endo-PG activity was estimated by measuring the decrease in reduced viscosity of 1.0 ml of enzyme or enzyme plus inhibitor in a 0.1% (wt/vol) solution of sodium polygalacturonate in 0.05 M sodium acetate, pH 5.0, at 30°C . A No. 75 Cannon-Manning Semi-micro Viscometer (water flow time of ≈ 130 s; Cannon Instrument Co., State College, PA) was utilized. Temperature was controlled at $30.07 \pm 0.02^{\circ}\text{C}$ in a viscometer bath (Cannon CT-500; Cannon Instrument). Employment of ≈ 5 nkat of endo-PG in the viscometric system resulted in a linear rate of decrease in reduced viscosity ($R^2 = 0.98 \pm 0.1$) over the first 15 min of the reaction.

Physical measurements. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Bio-Rad Mini-Protein III apparatus according to the procedure of Laemmli (26). Gels were stained for protein with Coomassie blue and for carbohydrate by the periodic acid-Schiff stain (19) with commercially prepared reagents (Pierce, Rockford, IL). Isoelectric focusing was performed on 18 cm Immobiline Dry Strips, pH 6 to 11, at 20°C with a Multiphor II IEF/Electrophoresis system according to the manufacturer's instructions (Amersham Pharmacia Biotech) or in vertical slab gels (8.3 by 7.3 cm by 0.75 mm) (4). Gel chromatography in 6 M guanidinium chloride (gdmCl) followed published procedures (31). Cross-linked 6% agarose (Amersham Pharmacia Biotech) equilibrated in 6 M gdmCl (Sigma, grade I) was employed in a Pharmacia column (1.6 by 100 cm). A flow rate of 1.4 g/h/cm² was used and ≈ 1 -g fractions were collected. Data were treated in the common fashion for estimating molecular weight and Stokes radius, R_s (16).

The absorptivity at 280 nm of CmPGIP was experimentally estimated by the procedure of Scopes (36). Absorbance readings were taken on a Perkin-Elmer Lambda 3B spectrophotometer (Perkin-Elmer Corp., Wellesley, MA) in 1 cm quartz cuvettes. The absorptivity was also predicted from the inferred amino acid composition by the equation of Pace et al. (34). Circular dichroism spectra were measured in the laboratory of J. Soulages, Oklahoma State University, with a Jasco J-715 instrument (Jasco Ltd., Japan). For spectra in the near UV, a CmPGIP concentration of 2.2 mg/ml ($A_{280} = 2.05$) in 0.1 M NaCl plus 0.05 M sodium acetate buffer, pH 5.0, was used in a 1 cm path length quartz cell. In the far UV, a cell path length of 0.1 cm was utilized. Protein concentrations of 0.22 and 0.044 mg/ml in 5 mM sodium acetate buffer plus 10 mM NaCl, pH 5.0, were employed for this wavelength region. The SELCON curve-fitting algorithm of Sreerama and Woody (38) was used to estimate the percentages of secondary structural elements.

Equilibrium analytical ultracentrifugation was performed on a Beckman XL-A instrument (absorption optics only) with an An-60 Ti rotor and a six channel epon-charcoal centerpiece held at 20°C (Beckman Coulter, Fullerton, CA). Equilibrium runs were performed at 17,000 and 25,000 rpm.

Mass spectrometry of CmPGIP was carried out by the Recombinant DNA/Protein Resource Facility at Oklahoma State University (Stillwater) on an Applied Biosystems PE-PRO MALDI-TOF mass spectrometry workstation (Applied Biosystems, Foster City, CA). Sinapinic acid was employed as the matrix. The instrument was operated in the linear mode. Spectra represented 20 spectra at 50 shots per spectrum. Mass of the intact CmPGIP was determined by internal calibration by employing bovine serum albumin (BSA)+1 ($M_r = 66,431$) and BSA+2 ($M_r = 33,216$) ions. Mass of the isolated CNBr-generated peptide was determined by internal calibration by employing thioredoxin+1 ($M_r = 11,675$) and apomyoglobin+1 ($M_r = 16,953$). Spectra were subjected to 17 point Gaussian smoothing and de-isotoping.

Chemical properties. Protein and peptide sequencing was performed on a Procise 491 automated protein sequencer (Applied Biosystems) by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Sequencing was repeated at least three times on each sample. Before sequencing, CmPGIP protein or peptides were reduced with 1,4-dithio-DL-threitol and alkylated with 4-vinylpyridine in 6 M gdmCl in 1 M Tris-HCl, pH 8.4.

CNBr cleavage was performed on 4.5 mg of CmPGIP in 0.3 ml of 70% formic acid with a ratio of ≈ 200 mol CNBr per mole CmPGIP (ultimately 50 mol CNBr/mole Met). The reaction proceeded in the dark at room temperature for 24 h. Reagents were removed by adding 10 volumes of deionized H₂O and lyophilizing the reaction mixture. The residue was dissolved in 6 M gdmCl and subjected to gel filtration on a calibrated column (1.6 by 95 cm) of Superdex 200 (Amersham Pharmacia Biotech) equilibrated in 6 M gdmCl. Individual peaks were pooled, desalted, and analyzed by SDS-PAGE before selection of fragments for sequencing.

Determination of CmPGIP sulfhydryl content was performed in 1% SDS in 0.1 M sodium phosphate buffer, pH 8.0, by the procedure outlined by Habeeb (21).

PCR sequencing. RNA was extracted from a 'Magnum 45' cantaloupe 5 days postanthesis. Total RNA was extracted using the TRIzol reagent (Gibco BRL Invitrogen Life Technologies). The RNA was reverse transcribed using the SOLIDscript Solid Phase cDNA Synthesis Kit (CPG Inc., Lincoln Park, NJ). The 5' end of the cantaloupe *pgip* gene was polymerase chain reaction (PCR) amplified by producing degenerate primers (synthesized by Gibco BRL Invitrogen Life Technologies) from the N-terminal and central peptide sequences determined from purified CmPGIP protein (described below in chemical properties). The product was then sequenced and internal primers were designed for use in 5' and 3' rapid amplification of cDNA ends (RACE; Gibco BRL Invitrogen Life Technologies). This procedure was performed to obtain the remainder of the 5' and 3' sequences.

All PCR reactions, excluding 5' RACE, were performed following standard protocols (5) and Ready-To-Go PCR Beads

(Amersham Pharmacia Biotech) and a MiniCycler Instrument (MJ Research, Waltham, MA). PCR sequencing was carried out by the Oklahoma State University Recombinant DNA Resource Facility. PCR samples were amplified and sequenced in the forward and reverse directions from at least two PCR reactions to check for any possible mutations introduced by the polymerase.

Northern analysis. Total RNA was isolated from the seed cavity of mature (35 days postanthesis) and immature (2 days postanthesis) 'Magnum 45' cantaloupe. Seeds were removed from the mature fruit but remained in the RNA sample of the immature fruit. One milliliter of TRIzol per 100 mg of sample was used following the company protocol. Both optional steps to reduce polysaccharide contamination were performed. Total RNA (15 μ g) from mature and immature cantaloupe fruit was run on a denaturing RNA gel and transferred to BrightStar-Plus positively charged nylon membrane (Ambion) with the NorthernMax kit (Ambion) according to the company's protocol. Visualization of the *pgip* message was performed with the BrightStar Psoralen-Biotin Non-isotopic Labeling Kit to label a full-length cantaloupe *pgip* sequence. The probe was amplified by PCR and sequenced for verification of the amplified product. Blots were washed under high stringency conditions and visualized using a Kodak Image Station 440CF (Eastman Kodak).

RESULTS

Extraction and purification of CmPGIP. Preliminary tests on young cantaloupe plants and cantaloupe fruit at various stages of maturity indicated that fruit at <10 day postanthesis were the richest source of CmPGIP activity (data not shown). As shown in the purification scheme summarized in Table 1, the batch pre-extraction of the tissue before solubilization of the CmPGIP afforded an almost 60-fold purification step with less than a 20% loss of PGIP activity. Seven protein peaks were obtained upon cation exchange chromatography of the CmPGIP extract, and one peak exhibited the vast majority of the PGIP activity, $\approx 98\%$, versus *P. cucurbitae* PG. This CmPGIP activity eluted in a somewhat broad, symmetrical peak at about 0.22 M NaCl. When the fractions from the peak of PGIP activity were pooled, concentrated, reequilibrated in starting buffer, and reapplied to the column, PGIP activity and protein eluted in a symmetrical peak at the same NaCl concentration. Rechromatography of the PGIP-containing fractions with a shallower gradient affected no additional resolution.

Gel filtration of the pooled CmPGIP fractions from ion exchange yielded a single symmetrical peak with little increase in specific activity of the preparation. At this point, the CmPGIP activity had been purified over 540-fold at a yield of 28%. SDS-PAGE of the preparation followed by staining for protein and for carbohydrate indicated that the electrophoretic component, pre-

TABLE 1. Purification of cantaloupe polygalacturonase-inhibiting proteins (CmPGIP) from immature cantaloupe fruit

Purification step	Volume (ml)	PGIP (units/ml) ^a	Total PGIP (units)	Protein (μ g/ml)	Total protein (mg)	Species activity (units/mg of protein)	Yield (%)
Extraction of 1.6 kg of fruit tissue with 2 liters of 0.05 M PO ₄ plus 0.6 M NaCl, pH 7.2	3,185	11.7	37,100	2,470	7,867	4.72	100
Extraction of residue from 1.6 kg of fruit tissue with 2 liters of 0.05 M PO ₄ plus 0.6 M NaCl, pH 7.2, after exhaustive preextraction ^b	2,000	15.4	30,900	0.056	112	276	83
Concentration of extract with Minitan and then Amicon membrane concentrators	14.9	1,540	23,000	4,295	64	359	62
Anion exchange chromatography on Bio-Rad MP25, peak eluting at 0.22 M NaCl in 0.05 M PO ₄ , pH 7.2	34	331	11,300	133	4.5	2,510	30
Gel filtration on P-100 in 0.2 M acetate, pH 5.0, and then concentration	5.0	2,070	10,400	800	4.0	2,590	28

^a Solutions were exchanged into 0.05 M sodium acetate buffer, pH 5.0, before assaying for PGIP activity.

^b Preextraction process: tissue preextracted five times, each time for 30 min with 2.1 liters of deionized H₂O at 7°C; once for 16 h with 2 liters of 0.05 M sodium phosphate buffer plus 0.1 M NaCl, pH 7.2, at 7°C; and finally three times, each time for 30 min with 2 liters of 0.05 M sodium phosphate buffer, pH 7.2, at 7°C.

sumably CmPGIP, was a glycoprotein (17). SDS-PAGE and Coomassie staining of successive dilutions of the preparation suggested that the CmPGIP was >90% pure (data not shown). Assays for other biological activities indicated the presence of trace levels of pectin methylesterase activity in the purified CmPGIP preparations, but no protease or pectin lyase activities were detected.

We found that CmPGIP was stable both in 0.05 M sodium phosphate buffer, pH 7.2, and in 0.05 M sodium acetate buffer, pH 5.0, from 4 to 10°C. However, upon freezing the protein, much of the inhibitor's activity was lost in the 0.05 M sodium phosphate. This inactivation/denaturation in the presence of sodium phosphate may be the result of significant pH changes during freezing and thawing (35). CmPGIP could be stored frozen at -20°C in 0.05 M sodium acetate buffer, pH 5.0, for up to a year without detectable loss in activity.

Functional properties. Figure 1 summarizes the interaction of CmPGIP with extracts of PG from six fungi, four of them known fungal pathogens of cantaloupe fruit, i.e., *P. cucurbitae*, *F. solani*, *F. verticillioides*, and *D. bryoniae*. CmPGIP inhibited PG activity from *F. solani*, *P. cucurbitae*, and *A. niger*, whereas little or no measurable inhibition could be achieved for *F. verticillioides*, *D. bryoniae*, and *Rhizopus* PG(s). *P. cucurbitae* PG purified by binding to agarose-CmPGIP (data not shown) was inhibited to 90% by CmPGIP in solution, although we were unable to achieve complete inhibition. The rate of decrease of reduced specific viscosity of 0.1% polygalacturonate in the presence of enzyme or enzyme plus inhibitor was also determined for each of the PGs from three

of the cantaloupe pathogens. Inhibitor was used at a level sufficient to achieve maximal inhibition of the enzyme in the reducing group-forming spectrophotometric assay. CmPGIP slowed the rate of viscosity reduction of substrate by 99% with *F. solani* extract, 93% with *P. cucurbitae* extract, and 0% with *D. bryoniae* extract. These data are consistent with CmPGIP being able to inhibit most or all of the endo-PG activity from *P. cucurbitae* and *F. solani*, but being totally ineffective against endo-PG from *D. bryoniae*. Heat denatured CmPGIP exhibited no inhibitory effect on the endo-PG activity of any of the three extracts.

Physical properties. Table 2 summarizes the physical properties determined for CmPGIP. The native molecular weight as estimated by sedimentation equilibrium or gel chromatography was the same as that measured under denaturing conditions by SDS-PAGE, gel chromatography in 6 M gdmCl, or MALDI-TOF MS. Together, these results indicate that CmPGIP is a monomeric protein in the native state. The $R_s/R_{min} = 1.28$ by gel chromatography in dilute buffer is consistent with CmPGIP being a globular protein. We observed that the Stokes radius, R_s , of the disulfide cross-linked randomly coiled form of CmPGIP, 5.45 nm, is only 20% less than that of the randomly coiled linear form (i.e., disulfide bonds reduced), 5.87 nm. Such behavior would suggest that there is limited or very localized restraint imposed on the polypeptide chain by intrachain disulfide bonds.

Efforts to experimentally estimate the pI of CmPGIP on commercial gels and on gels that we prepared were not successful. Thus, we can only report that the pI predicted from the amino acid sequence of mature CmPGIP is 8.2. The absorptivity of CmPGIP used for concentration determinations, 0.93 ml/mg, was determined by the method of Pace et al. (34) from the inferred amino acid sequence of CmPGIP. The experimentally estimated value (36), 0.72 ml/mg, was deemed less reliable because of its susceptibility to error-inducing factors such as stray light or errors in the wavelength setting.

Figure 2 presents the near- and far UV circular dichroic spectra of CmPGIP. Overall, the far UV circular dichroism (CD) spectrum suggests that any periodic secondary structure of the protein is dominated by β -sheet. Prediction of the proportions of secondary structures from the spectrum suggests around 30% β -sheet structure and as much as 23% turns. This is in reasonable agreement with values recently reported for *Phaseolus vulgaris* (bean) PGIP (44) of 34.6% β -strand and 21.5% turns. The inset of Figure 2 shows the near UV CD spectrum. It would appear that the signal is dominated by Tyr (2,6) residues and disulfide bond(s) (6) with a Trp signal visible at ≈ 295 nm (2).

Fig. 1. Interaction of cantaloupe polygalacturonase-inhibiting proteins (CmPGIP) with PGs from selected fungi. Various levels of pure CmPGIP were incubated with crude extracts of fungal lesions or commercial PG preparations, all at constant levels of PG activity. Open circles, *Phomopsis cucurbitae* PG purified on a CmPGIP affinity column; open squares, *Fusarium solani* lesion extract; open triangles, commercial *Aspergillus niger* preparation; open diamonds, *F. verticillioides* lesion extract; closed circles, *P. cucurbitae* lesion extract; closed squares, *Didymella bryoniae* lesion extract; and closed triangles, commercial *Rhizopus* sp. preparation. The experimental points for *P. cucurbitae* purified PG, *P. cucurbitae* lesion extract, *F. solani* lesion extract, and *A. niger* PG were individually fit with curves of the following form: $P_w = (P_{init} - P_{min}) \times [\exp(-k \times w)] + P_{min}$, where P_w is the observed PG activity at w pmoles of PGIP, P_{init} is the observed PG activity in the absence of PGIP, P_{min} is the residual reducing activity at maximal PGIP inhibition, w is the pmoles of PGIP added, and k is an empirically derived constant to give the best fit of the curve to the data. No physical significance is implied for the constant k . The experimental points for *D. bryoniae*, *F. verticillioides*, and *Rhizopus* sp. PGs are fit with straight lines of slope = 0.

TABLE 2. Physical properties of cantaloupe polygalacturonase-inhibiting proteins (CmPGIP) from cantaloupe fruit

Property	Value
Native molecular weight by sedimentation equilibrium in dilute buffer ($v = 0.734$)	37,500 $\pm$ 700
R_s by gel chromatography in dilute buffer	2.86 nm
R_s/R_{min} by gel chromatography	1.28
Molecular weight by SDS-PAGE	39,400 $\pm$ 2,000
Molecular weight by gel chromatography in 6 M gdmCl	38,000 $\pm$ 2,000
Molecular weight by MALDI-TOF MS	38,431
Molecular weight of mature polypeptide chain inferred by cDNA sequence	34,346
Apparent percentage of attached oligosaccharides	10.6%
R_s of random coil in 6 M gdmCl	5.87 $\pm$ 0.15 nm
R_s of S-S cross-linked random coil in 6 M gdmCl	5.45 $\pm$ 0.15 nm
pI (estimated)	8.2
Absorptivity at 280 nm	0.93 ml/mg
Secondary structure estimated by CD	
α -Helix	11 $\pm$ 2%
β -Sheet, antiparallel	25 $\pm$ 5%
β -Sheet, parallel	7 $\pm$ 2%
Turn	23 $\pm$ 3%
Other	38 $\pm$ 5%

Chemical properties. N-terminal sequencing of CmPGIP yielded the following sequence: Glu-Leu-Cys-His-Pro-Asn-Asp-Lys-Glu-Val-Leu-Leu-Asn-Ile-Lys-Ala-Phe-Asn-Asn-Pro-Tyr-Ile. CmPGIP was cleaved at Met residues by treatment with CNBr. Cleavage products were then separated by size by gel chromatography in 6 M gdmCl. Four peaks were obtained with the following Stokes radii: 6.1, 4.7, 3.5, and 2.7 nm. These fractions were further evaluated by SDS-PAGE. Material from the first three peaks exhibited multiple bands. Only a single band of $M_r = 12,600$ by SDS-PAGE was observed for the 2.7 nm peak while its estimated M_r by gel chromatography in 6 M gdmCl was $\approx 9,500$. This differential behavior between the two methods suggested that oligosaccharide was covalently attached to the polypeptide chain. This CNBr peptide was analyzed by MALDI-TOF MS. Its mass spectrum gave rise to a predominant peak of 9,881 m/z and smaller peaks at 8,883, 9,746, and 10,043 m/z . The mass difference between the major species and the species at 10,043 is 162 and is suggestive of a hexose residue difference, whereas the difference between the major species and that at 9,746 is suggestive of a pentose residue difference (33). Each of the aforementioned mass species also had a shadowing peak of 28 mass units greater mass. We suspect these to be formulation derivatives of their parent species. The 2.7 nm CNBr peptide was subjected to N-terminal sequencing and the following sequence was obtained: Gly-Lys-Val-Asp-Phe-Asn-Tyr-Ile-Asp-Leu-Ser-Arg-Asn-Lys-Leu-Val-Gly-Asp-Gly-Ser-Leu-Ile. This sequence aligns with a PGIP region starting at residue 195 of bean PGIP. The exact position number differs among PGIPs from different sources. These two amino acid sequences from two widely separated regions of the protein were subsequently used to design oligonucleotides for amplification of *pgip* for sequencing.

***pgip* sequence.** Using the PCR techniques mentioned previously, we sequenced 992 nucleotides of *Cm_{pgip}* cDNA that contained a complete open reading frame of 978 nucleotides and encoded 326 amino acids. The complete nucleotide and amino acid sequences for *Cm_{pgip}* can be viewed at GenBank (accession no. AY288911). A comparative alignment is shown in Figure 3. The predicted molecular weight of the full-length protein is 36,612 and 34,346 for the mature protein. The leader sequence for CmPGIP is Met-His-Ser-His-Asn-Leu-Leu-Leu-Leu-Phe-Phe-Phe-Phe-Thr-Val-Ser-Phe-Ala and is cleaved off to make the mature protein. This leader sequence is similar to the leader sequences from some of the other cloned PGIPs (9). A BLAST search with the mature CmPGIP revealed high similarity to other PGIP homologues, with apple (*Malus domestica*) (42) having the highest identity (68% over the entire length of the mature protein).

***pgip* message.** As illustrated in Figure 4, northern blot analysis of total RNA isolated from mature (35 days postanthesis) and immature (2 days postanthesis) cantaloupe fruit tissue revealed a band of 1.25 kb in immature fruit and no detectable band in mature fruit. The 1.25-kb message is consistent with a 978-bp predicted open reading frame plus 5' and 3' untranslated regions.

DISCUSSION

To our knowledge, this is the first report of the isolation and characterization of a cucurbit PGIP. CmPGIP is obtained through a 550-fold purification from immature cantaloupe fruit. Our molecular weight measurements under native and denaturing conditions unequivocally demonstrate that CmPGIP is a monomeric, globular protein in its native state. The far UV CD spectrum of CmPGIP is qualitatively and quantitatively similar to that for bean PGIP (32) and suggests β -sheet is the predominant type of periodic secondary structure. Secondary structural predictions for the CmPGIP polypeptide chain from its amino acid sequence by two different algorithms (10,18) did not yield a clear consensus of locations of each type of periodic structure. Both methods did

predict, however, a markedly higher proportion of β -sheet/ β -turn structures than of α -helix, and this is consistent with the far UV CD spectrum. This is the first published spectrum of the near-UV CD of a PGIP. The moderately strong signals for Tyr and Trp in this region suggest that it may offer a possible spectral target for study of the microenvironmental changes of selected aromatic side chains in PGIP as a result of the interaction between PG and PGIP.

The hydrodynamic behavior of S-S cross-linked randomly coiled CmPGIP compared with that of its randomly coiled linear form is consistent with the disulfide bonds in the chain occurring between cysteines relatively close to one another in the linear sequence (41). Our observation is consistent with the location of disulfide bonds in *Phaseolus vulgaris* PGIP determined by mass spectra/proteolytic digestion (32). Although the inferred sequence of CmPGIP requires that the protein possess one free sulfhydryl (Cys 38), chemical modification suggested only ≈ 0.2 sulfhydryls per molecule. Either not all of the population of sulfhydryls was available for modification in SDS or the gene sequence that we determined is for a different PGIP that has a Cys in that position whereas the protein we isolated does not. As with nearly all PGIPs, eight of the nine Cys are conserved in the sequence (Fig. 3) which further suggests that the CmPGIP is disulfide constrained in a manner identical to bean PGIP. This appears not to be the case for all PGIPs, however. In eucalyptus, the mature polypeptide chain begins after Cys 3, and Cys 306 is substituted (9). In *Citrus sinensis*, *Fortunella margarita*, and *Poncirus trifolata* (alignments provided in literature citation 13), the polypeptide chain terminates before Cys 306. This means that one or two of the S-S bonds are likely absent in these inhibitors, and according to their inferred amino acid sequences, they each should possess

Fig. 2. Circular dichroism spectrum of cantaloupe polygalacturonase-inhibiting proteins (CmPGIP). The far UV CD spectrum was measured at 25°C with protein concentrations of 0.22 and 0.044 mg/ml in 5 mM sodium acetate buffer plus 10 mM NaCl, pH 5.0, in a 0.1 cm path length quartz cell. Inset: The near UV CD spectrum of CmPGIP at 25°C in a 1 cm path length quartz cell at a protein concentration of 2.2 mg/ml in 0.1 M NaCl plus 0.05 M sodium acetate buffer, pH 5.0.

two free sulfhydryls. The sequence fidelity for the 10.5 LRRs postulated for PGIP (12,39) is maintained in CmPGIP. Positions containing met are not conserved among the various PGIPs. Only one of four mets in mature CmPGIP is in a homologous position with those of its closest PGIP homologs.

Three potential N-glycosylation sites are predicted from the sequence of CmPGIP. They are at Asn 83, Asn 231, and Asn 268. Asn 231 resides within the peptide that we isolated from CNBr cleavage of CmPGIP. Mass spectral measurements suggest the nominal mass of that (glyco)-polypeptide to be 9,881. The M_r of the polypeptide portion calculated from its amino acid sequence (Fig. 3) is 8,588. This yields a mass difference of 1,293. This mass difference would be sufficient to accommodate the common

oligosaccharide core structure of glycoproteins, GlcNac₂Man₃, but is not of sufficient magnitude, 1,373, to be equivalent to the oligosaccharide attached to bean PGIP (32). The total mass difference between that of the predicted polypeptide chain of CmPGIP and its total mass by MALDI-TOF MS is 4,085 and is likely to represent attached carbohydrate. If the apparent nominal mass of the oligosaccharide attached at Asn 231 is subtracted from the total mass of 4,085, the difference is 2,792. This mass of putative carbohydrate could accommodate two oligosaccharides of size similar to those attached to bean PGIP and suggests that Asn 83 and Asn 268 of CmPGIP may be glycosylated.

Like other PGIPs, CmPGIP appears to bind specifically to endo-PGs. CmPGIP inhibits PGs from two natural fungal patho-

CmPGIP	E L C H P N D K E V L L N I K K A F N N P Y I L T S W K P E E D C C T - - W Y C	38
PcPGIP	D L C N P D D K K V L L Q I K K A F G D P Y V L A S W K S D T D C C D - - W Y C	38
LePGIP	V R C N P K D K K V L L Q I K K D L G N P Y H L A S W D P N T D C C Y - - W Y V	38
GmPGIP	E L C N P Q D K Q T L L Q I K K E L G N P T T L S S W H P K T D C C N N S W V G	40
PvPGIP2	E L C N P Q D K Q A L L Q I K K D L G N P T T L S S W L P T T D C C N R T W L G	40
CmPGIP	V E C D L K S H - - R I I A L T I F A D D E L S G P - - I P P F V G D L P F L E	74
PcPGIP	V T C D S T T N - - R I N S L T I F A G Q - V S G Q - - I P A L V G D L P Y L E	73
LePGIP	I K C D R K T N - - R I N A L T V F Q A N - I S G Q - - I P A A V G D L P Y L E	73
GmPGIP	V S C D T V T P T Y R V D N L D L - S E L N L R K P Y P I P P S V G S L P C L K	79
PvPGIP2	V L C D T D T Q T Y R V N N L D L - S G L N L P K P Y P I P S S L A N L P Y L N	79
CmPGIP	N L M F H K L P N L T G P I P P T I A K L H N L K Y L D L S W N G L S G P I P S	114
PcPGIP	T L E F H K Q P N L T G P I Q P A I A K L K G L K S L R L S W T N L S G S V P D	113
LePGIP	T L E F H H V T N L T G T I P P A I A K L T N L K M L R L S F T N L T G P I P E	113
GmPGIP	F L Y I T N N P N I V G T I P T T I T K L T K L R E L N I R Y T N I S G Q I P H	119
PvPGIP2	F L Y I G G I N N L V G P I P P A I A K L T Q L H Y L Y I T H T N V S G A I P D	119
CmPGIP	F L G S L S N L D I L D L S F N R F T G S I P S S L A N L R R L G T L H L D R N	154
PcPGIP	F L S Q L K N L T F L D L S F N N L T G A I P S S L S E L P N L G A L R L D R N	153
LePGIP	F L S Q L K N L T L L E L N Y N Q F T G T I P S S L S Q L P N L L A M Y L D R N	153
GmPGIP	F L S Q I K A L G F L D L S N N K L S G N L P S W L P S L P D L Y G I S F D N N	159
PvPGIP2	F L S Q I K T L V T L D F S Y N A L S G T L P P S I S S L P N L V G I T F D G N	159
CmPGIP	K L T G P I P D S F G N F K G - K V P Y L Y L S H N Q L S G K I P T S M G K V D	193
PcPGIP	K L T G H I P I S F G Q F I G - N V P D L Y L S H N Q L S G N I P T S F A Q M D	192
LePGIP	K L T G T I P E S F G R F K G P N I P D L Y L S H N S L T G H V P A S L G D L N	193
GmPGIP	Y I S G P I P D L F A S V - S E R F G F I S L S G N R L I G K I P A S L G K P D	198
PvPGIP2	R I S G A I P D S Y G S F - S K L F T S M T I S R N R L T G K I P P T F A N L N	198
CmPGIP	F N Y I D L S R N K L V G D G S L I F G S K K T T E I V D L S R N L L E F N I S	233
PcPGIP	F T S I D L S R N K L E G D A S V I F G L N K T T Q I V D L S R N L L E F N L S	232
LePGIP	F S T L D F S R N K L E G D V S F L F G K N K T S Q V I D L S R N L L E F N I S	233
GmPGIP	M K I V D L S R N M L E G D A S V L F G S E K H T E R I Y L A N N L F A F D L G	238
PvPGIP2	L A F V D L S R N M L E G D A S V L F G S D K N T Q K I H L A K N S L A F D L G	238
CmPGIP	K V V F P R T L T W L D L N H N K I F G E I P T G V V K L E - L Q M L N V S Y N	272
PcPGIP	K V E F P T S L T S L D I N H N K I Y G S I P V E F T Q L N - F Q F L N V S Y N	271
LePGIP	K S E F A E S L I S L D L N H N R I F G S L P P G L K D V P - L Q F F N V S Y N	272
GmPGIP	K V R L S K T L G L L D V G H N L I Y G T L P K G L T S L K D L Y Y L D V S Y N	278
PvPGIP2	K V G L S K N L N G L D L R N N R I Y G T L P Q G L T Q L K F L H S L N V S F N	278
CmPGIP	A L C G R I P M G G K L Q S F D V Y S Y F H N K C L C G K P L G S C K	307
PcPGIP	R L C G Q I P V G G K L Q S F D E Y S Y F H N R C L C G A P L P S C K	306
LePGIP	R L C G Q I P Q G G T L Q S F D I Y S Y L H N K C L C G S P L P K C K	307
GmPGIP	N L C G E I P R G G K L Q E F D A S L Y A N N K C L C G S P L P S C T	313
PvPGIP2	N L C G E I P Q G G N L Q R F D V S A Y A N N K C L C G S P L P A C T	313

Fig. 3. Comparison of cantaloupe polygalacturonase-inhibiting proteins (CmPGIP) amino acid sequence with other PGIP sequences. The predicted amino acid sequences are given in the single letter code. Sequences aligned are CmPGIP, *Cucumis melo*; P-PGIP, *Pyrus communis* (40); LePGIP, *Lycopersicon esculentum* (39); GmPGIP, *Glycine max* (29); and PvPGIP2, *Phaseolus vulgaris* PGIP2 (28). The amino acid sequences were aligned using MegAlign 4.05 software (DNASTAR Inc., Madison, WI). Conserved regions are shaded.

gens of cantaloupe as well as that of *A. niger*. *Rhizopus* sp., *D. bryoniae*, and *F. verticillioides* PGs are inhibited very little or not at all by CmPGIP. *D. bryoniae* is a cantaloupe pathogen that is more likely to attack immature cantaloupe fruit (44) and thus its endo-PG might be anticipated to be unaffected by CmPGIP. Furthermore, it has been reported that *D. bryoniae* PG isozymes obtained from lesions on immature cantaloupe fruit are predominantly exo-PG (44), whereas *P. cucurbitae* (45) and *F. solani* (46) exhibit appreciable endo-PG activities. Also of interest is the fact that CmPGIP, like PGIPs from a number of other plants, does not inhibit PG from *F. verticillioides* (previously known as *F. moniliforme*) (summarized in 13). Leckie et al. (28) elegantly demonstrated with bean PvPGIP2 and an *F. verticillioides* PG that position 219 (numbering of Figure 3) is intimately involved in its recognition and binding to PG. Pear, tomato, and grape PGIPs have been shown to be ineffective against *F. verticillioides* PG (summarized in literature citation 13). The amino acid sequences of CmPGIP and the aforementioned PGIPs are highly variable over the linear sequence 10 residues either side of position 219 and exhibit little sequence homology with bean PvPGIP2. These observations suggest that this linear region of PGIP is prominent in the PG-binding domain of PGIP.

Northern blot analysis on total RNA isolated from immature cantaloupe fruit tissue revealed one mRNA of 1.25 kb. Interestingly, mature cantaloupe produced no detectable levels of *pgip* message, yet the mature fruit has measurable PGIP activity, albeit to a lesser extent than the immature fruit. This suggests one of three possibilities. First, the sensitivity of our northern blots was not high enough to detect the small amount of *pgip* message produced in mature cantaloupe fruit. Second, the protein found in mature fruit is a result of mRNA produced early on in the fruit maturation process. Third, the PGIP activity detected in the mature fruit is from a different gene product than we screened for by northern blots. Since immature fruit had a significant amount of

pgip message and contained immature seed tissue, and the mature fruit sample did not contain seed tissue or detectable levels of mRNA, it is not inconceivable that the seed tissue produces high levels of *pgip* message. We plan to test this further.

In light of the fact that *pgip* gene families have clearly been shown to exist (13), as well as have expression of two or more of their gene products (1,14), we feel it necessary to offer a caveat to our interpretation of the results we report on CmPGIP. Amino acid substitutions of only 1 to 3 positions out of 332 have been shown to change the functional properties of bean PGIP without changing its macromolecular physical and chemical properties (28,32). Thus, it may be that the PGIP protein that we have isolated and characterized is not the product of the *pgip* gene we have cloned. It may instead be the product, or products, from one or more different members of the *Cm**pgip* gene family.

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Fig. 4. Northern blot analysis of cantaloupe *pgip*. **A**, Denaturing RNA gel of 2 μ g Millennium mRNA size standard (S) for comparison to 15 μ g of total RNA from mature (M) and immature (I) cantaloupe fruit. **B**, Northern blot of cantaloupe polygalacturonase-inhibiting proteins (CmPGIP) message. This figure is representative of three northern blot experiments.

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