European Journal of Plant Pathology (2005) 111: 67–76

Polygalacturonase-inhibiting protein activity in cantaloupe fruit as a function of fruit maturation and tissue origin★

Wayne W. Fish
USDA-ARS, SCARL, P.O. Box 159 Lane, OK 74555, USA (Phone: +1-580-889-7395; Fax: +1-580-889-5783; E-mail: wfish-usda@lane-ag.org)

Accepted 20 June 2004

Key words: *Aspergillus niger*, cantaloupe fruit development, *Didymella bryoniae*, *Fusarium solani*, PGIP activity, *Phomopsis cucurbitae*, polygalacturonase

Abstract

Netted cantaloupe (*Cucumis melo* var. *cantalupensis* cv. Magnum 45) were harvested from 5 to 35 days postanthesis. The fruit of each age group were divided into exocarp, outer mesocarp, mid mesocarp, inner mesocarp, placenta, and seed. Each tissue was extracted and assayed for polygalacturonase-inhibiting protein (PGIP) activity against polygalacturonases (PGs) from three fungal pathogens of cantaloupe fruit. The PGIP activity of all tissues except placenta was high from the flower stage through the first week of fruit development but decreased markedly between 5 and 10 days postanthesis. PGIP activity against *Phomopsis cucurbitae* PG remained high and nearly constant in placental tissue throughout fruit development. However in this same tissue, PGIP activity against *Fusarium solani* PG decreased during fruit development to about 25% of its level in the 5-day-old fruit. This differential change in PGIP activity toward the two PGs suggests that different forms of the inhibitor are expressed between early and late stages of cantaloupe fruit development. The results also illustrate the importance of using multiple pathogen enzyme systems that can provide an opportunity for more accurate elucidation of mechanisms involved in the host–pathogen interaction.

Abbreviations: AEBSF – 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; BSA – bovine serum albumin; EDTA – ethylenediaminetetraacetic acid; PG – polygalacturonases; PGA – polygalacturonic acid; PGIP – polygalacturonase-inhibiting protein.

Introduction

During pathogenesis, fungi must breech the plant cell wall. This is accomplished by secretion of a cadre of hydrolytic enzymes whose substrates are plant cell wall constituents (DeLorenzo et al., 1997). Among these enzymes are endopolygalacturonases (PGs) that target the polygalacturonic acid regions of pectin. Polygalacturonase inhibitor proteins (PGIPs) are glycoproteins that are localized in the plant cell wall and inhibit fungal PGs (DeLorenzo et al., 2001; DeLorenzo and Ferrari, 2002; D'Ovidio et al., 2004). Their role as part of

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the plant defense system apparently involves inhibiting PG activity and favoring the release of oligogalacturonide (OG) fragments of 10–15 residue chain length that elicit a variety of plant defense responses (Cervone et al., 1989; Darvill et al., 1992; Ridley et al., 2001). PGIPs have been found in almost every plant species investigated, although dicotyledonous plants have been the most widely studied (summarized in DeLorenzo et al., 2001; D'Ovidio et al., 2004).

Fungal PG–plant PGIP interactions are highly discriminating. PGIPs from various plant sources differ in their ability to inhibit PGs from different fungi, and PGIPs from a single plant species have different affinities for PGs from various fungi or iso-forms from the same fungus (Abu-Goukh and Labavitch, 1983; Lafitte et al., 1984; Yao et al., 1999). A striking example of the specificity and affinity for fungal PGs was provided with bean PGIPs (Leckie et al., 1999). The authors showed that a single mutation of a lysine at position 253 of PvPGIP1 into a glutamine at that position in PvPGIP2 is sufficient to confer the capacity to interact with both *F. moniliforme* PG and *A. niger* PG. With lysine at that position, as in PvPGIP1, the inhibitor can interact only with *A. niger* PG.

Pgip genes have been cloned from a large number of plant species where they usually exist as gene families (DeLorenzo et al., 2001; DeLorenzo and Ferrari, 2002; D'Ovidio et al., 2004). At least two genes have been reported for several plant species, and at least five *pgip* genes exist for *Phaseolus vulgaris*. Expression of individual genes appears to be regulated in a manner unique to each gene (Devoto et al., 1997, 1998). The level of expression of PGIPs in a plant is regulated by tissue type, tissue development, and stress stimuli (DeLorenzo et al., 2001; DeLorenzo and Ferrari, 2002; D'Ovidio et al., 2004). In fruit, levels of PGIP activity are generally higher in the immature stage than in the mature state (Abu-Goukh et al., 1983; Johnston et al., 1993; Yao et al., 1999). These higher levels of PGIP activity seem to correlate with greater resistance to fungal attack. For example, increasing susceptibility of ripening pear fruit to *B. cinerea* correlates with a decline in the concentration of PGIP (Abu-Goukh et al., 1983), and immature green raspberry fruit, the stage at which PGIP levels are maximal, are more resistant than mature fruits to fungal attack (Johnston et al., 1993). Details on individual *pgip* gene expression as a function of fruit development and information about levels of PGIP expression among fruit tissue types are limited.

Much of the information about PG–PGIP interactions comes from studies involving agronomically important crop plants and their respective fungal pathogens. Interestingly, there is little information about cucurbit PGIPs, their interactions with fungal pathogen PGs, and their expression and control. Of particular interest is the nature of the netted cantaloupe (*Cucumis melo* var. *cantalupensis*) fruit PGIP (CmPGIP) and its interaction with PGs from those fungi that cause fruit decay in cantaloupe at selected stages of fruit development. Early reports suggested the presence of PGIP activity in cucurbits (Skare et al., 1975; Lafitte et al., 1984), and a recent report describes the isolation, characterization, and gene sequence of PGIP from immature cantaloupe fruit (Fish and Davis, 2004). CmPGIP from immature cantaloupe fruit possesses physical/chemical properties similar to previously studied PGIPs. It is a glycoprotein of 38,500 molecular weight, possesses considerable β-sheet secondary structure, and is minimally constrained by localized disulfide bonds. Its sequenced open reading frame predicts a mature protein of 307 amino acids with up to 68% identity to PGIPs from other plants and the presence of ten modules of leucine-rich repeats (LRR's) (Fish and Davis, 2004).

As a natural extension of the molecular characterization of PGIP from immature cantaloupe fruit, it was desirous to learn more about the expression of PGIP activity by the fruit during its development. Because of its larger size, cantaloupe fruit can be more easily investigated with respect to changes in PGIP activity in individual tissues than can smaller fruit. Thus, the purpose of this research was to examine levels of PGIP activity in cantaloupe fruit as a function of fruit maturation and fruit tissue origin. Levels of CmPGIP were measured by comparing its activity against PGs from each of three cantaloupe fruit decay pathogens that exhibit different modes of pathogenesis.

Materials and methods

Reagents

Sodium 4-(2-Aminoethylamino)-1-naphthalenesulfonic acid (AEBSF), ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA), and

polygalacturonic acid from oranges (PGA) were purchased from Sigma (St Louis, MO). The derivatization agent for reducing-group quantification (Gross, 1982), 2-cyanoacetamide, was purchased from Acros Organics (Geel, Belgium). All other commonly used buffer salts, acids, and bases were reagent grade.

Source material for polygalacturonase (PG) enzymes

The fungi, *Phomopsis cucurbitae*, isolate OK-1062; *Fusarium solani*, isolate OK-737; and *Didymella bryoniae*, isolate TX97-128, were the sources of PG enzymes from cantaloupe fruit pathogens and were obtained from Dr Benny Bruton, USDA, Lane, OK. *Aspergillus niger* PG was from a commercial source (Sigma, St Louis, MO) and was used without further purification. The *A. niger* PG was included to allow a comparison of CmPGIP efficacy to other plant PGIPs since it is one of the more widely used PG enzymes to test PGIP activity (DeLorenzo et al., 2001). PG was prepared from fruit lesions produced by each of the fungi by culturing each and inoculating cantaloupe fruit according to published procedures (Zhang et al., 1997; Zhang and Bruton, 1999; Zhang et al., 1999). Mature, full-slip fruit were inoculated with *F. solani* or *P. cucurbitae* while 10 day postanthesis fruit were used for *D. bryoniae*. Lesions were 6–8 days old when harvested. A crude enzyme extract was prepared from lesions of each organism by grinding the lesion 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-fold for *P. cucurbitae* and ~70-fold for *D. bryoniae* and *F. solani* on an Amicon YM-10 membrane (Millipore Corp., Bedford, MA). It was subsequently exchanged into 0.05 M sodium acetate buffer, pH 5.0, by diafiltration while in the same apparatus. The diafiltration also facilitated removal of uv-absorbing small molecules that when not removed, reduced the precision of the enzyme assay by their interfering absorbance.

Preparation of cantaloupe fruit PGIP

Cantaloupe fruit (cv. Magnum 45) were from the 2000, 2001 and 2002 crops at the Lane Ag Center, Lane, OK. Female flowers were tagged on the day they opened. Fruit from the 2000 crop were harvested every 10 days from 10 days postanthesis to 50 days postanthesis (full slip plus 10 days). Fruit from the 2000 crop were separated into inner-, mid-, and outer-mesocarp, and exocarp. Fruit from the 2001 crop were harvested at 5, 10, 15, 20, 30 and 35 days (full slip) postanthesis. Five-day melons were separated into exocarp, mesocarp, and seeds + seed cavity. Fruit at all later stages of development were divided into exocarp, outer mesocarp, mid mesocarp, inner mesocarp, seed cavity (placental material), and seed. Female flowers at anthesis and 1-day-old fruit were collected from the 2002 crop. Samples were frozen at −20 °C until extracted for PGIP assay.

Tissue samples were extracted by combining a given weight of thawed sample with a volume of pre-extraction buffer equal to the tissue sample weight. For mesocarp samples, 52.5 g tissue were utilized. Samples of 26.25 g were taken for 5-day-old fruit, exocarp, seed, and seed cavity material. Pre-extraction buffer was 1 mM EDTA + 5 μM AEBSF in 0.05 M sodium acetate buffer, pH 5.0. Samples in pre-extraction buffer were ground in a blender for 1 min followed by homogenization for 1 min (Brinkman Polytron Homogenizer, Westbury, NY). The homogenate was centrifuged at $16{,}000 \times g$ at 15 °C for 1 h in a GSA rotor with a Sorvall RC-5B centrifuge. The supernatant was discarded and 25 ml of 1 M NaCl + 1 mM EDTA + 5 μM AEBSF in 0.05 M sodium acetate buffer, pH 5.0 were added to the residue and stirred 30 min at room temperature. This mixture was centrifuged at $16{,}000 \times g$ at 15 °C for 1 h and the supernatant set aside. The above extraction/centrifugation procedure was repeated on the residue, and the second supernatant was combined with the first. A third extraction of the residue indicated that ≥94% of the PGIP activity was recovered in the first two extracts, so two extractions were routinely employed. Tests with tissue from 15-day-old fruit demonstrated that less than 10% of the tissue PGIP was lost in the pre-extraction. The CmPGIP extract was filtered through Whatman #1 filter paper and centrifuged at $16{,}000 \times g$ at 15 °C for 1 h to remove all particulate matter. The CmPGIP extract was then concentrated to 2.5 ml with a Centriprep YM-10 centrifugal filter device (Millipore Corp., Bedford, MA). The 2.5 ml of concentrated PGIP extract were exchanged into a 0.6 M sodium acetate buffer, pH 5.0 by gel

chromatography with the aid of a Sephadex PD-10 column (Sigma, St Louis, MO). The CmPGIP from the starting weight of tissue thus ended in 3.5 ml total volume. To normalize the results to a tissue dry weight basis, fresh cantaloupe tissues were dried at 80 °C until they reached a constant weight for two successive weighings which was normally ~36 h.

Protein concentrations were estimated by the method of Bradford (1976) using a commercial kit (Bio-Rad Laboratories, Richmond, CA).

PG and CmPGIP activity assays

Inhibition of PG activity by CmPGIP-containing extracts was determined by conducting the procedure with and without various amounts of extract pre-incubated with a given PG. Approximately 5–7 nanokatals (nkat) of PG activity from each fungus were employed in each assay. Healthy cantaloupe fruit tissue extracts exhibited no detectable PG activity. Pre-incubations of PG alone or with various levels of CmPGIP-containing extract were carried out in a total volume of 0.45 ml. This volume consisted of 150 μl 0.05 M sodium acetate buffer + 315 μg BSA, pH 5.0, 100 μl PG in 0.05 M sodium acetate buffer, pH 5.0, and 200 μl of various levels of CmPGIP in 0.6 M sodium acetate, pH 5.0. Supplemental experiments showed that the final concentration of sodium acetate, 0.29 M, in the pre-incubation solution did not inhibit the PG–CmPGIP interaction. Apparent inhibition of the PG–PGIP interaction began to occur at 0.32 M sodium acetate, pH 5.0. After pre-incubation at room temperature for 15 min followed by pre-incubation at 30 °C for 5 min, 1.35 ml of a solution that contained 0.133% PGA + 700 μg ml^{-1} BSA in 0.05 M sodium acetate, pH 5.0 (pre-equilibrated to 30 ± 1 °C) were added to the pre-incubation mixture to initiate the reaction. Duplicate 200 μl aliquots were removed from the incubation mixture at 0, 2.5, 5.0, and 7.5 min and added to 1.2 ml of the stop buffer/derivatization reagent. The stop buffer/derivatization system to quantify reducing groups released from sodium polygalacturonate was that of Gross (1982). D-galacturonic acid was used as the standard to convert absorbance change at 276 nm to molar concentration of reducing groups formed. The R^2 value for a linear least squares fit to the eight points collected in this discontinuous assay routinely ran 0.99 ± 0.01. In some instances, endo PG activity was estimated by measuring the decrease in reduced viscosity at 30 °C of 1.0 ml of enzyme or enzyme plus inhibitor in a 0.1% (w/v) solution of sodium polygalacturonate in 0.05 M sodium actate, pH 5.0. A No. 75 Cannon–Manning semi-micro viscometer (water flow time ~130 s) (Cannon Instrument Co., State College, PA) was utilized. Temperature was controlled at 30.07 ± 0.02 °C in a Cannon CT-500 viscometer bath. Employment of 4–5 nkat of endo PG 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.

Calculations employed to estimate CmPGIP activity

Following precedence (Albersheim and Anderson, 1971; Cervone et al., 1987; Stotz et al., 1993; Yao et al., 1995), one unit of CmPGIP activity was defined as the amount of inhibitor required to reduce one unit of PG activity by 50%. Because the assays did not employ individually pure enzymes and inhibitors, it was anticipated that reducing group formation would not likely be the result of *endo* PG activity only and that 100% inhibition of reducing group formation would not be observed. Such turned out to be the case as illustrated for each of the fungal lesion extracts in Figure 1. The residual reducing group-forming activity still remaining after the maximum level of PG had been inhibited necessitated the employment of a reliable empirical means to precisely determine the amount of cantaloupe tissue extract that produced a 50% inhibition (of presumably *endo* PG activity) in any given assay. A relationship derived by Fish and Madihally (2004) was employed for this purpose. Fixed levels of PG were incubated with varying volumes of PGIP-containing extract and the level of reducing group-forming activity (i.e. PG activity) was measured. Ever-increasing volumes of PGIP-containing extract were employed until no further decrease was observed in the rate of reducing group formation. Quantification of PGIP activity employed the following approach:

$$\text{Fractional PG activity remaining} = \text{Fxr}(\text{PG}_\text{V}) = \frac{(\text{PG}_\text{V}) - (\text{PG}_\text{min})}{(\text{PG}_0) - (\text{PG}_\text{min})} \quad (1)$$

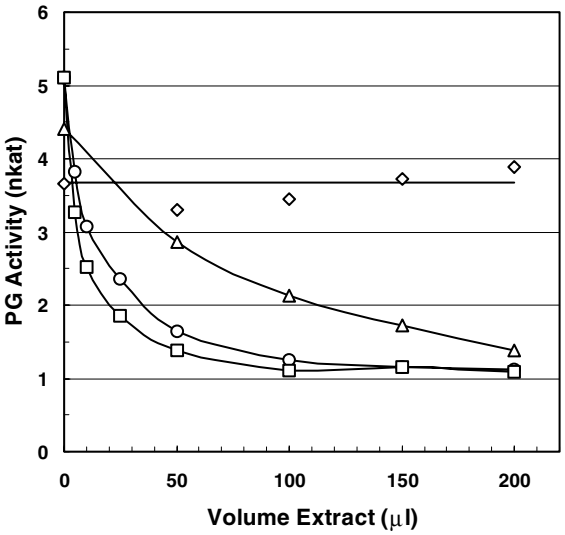

Figure 1. Interaction of cantaloupe PGIP from 10-day-old fruit with PGs from selected fungi. Various volumes of extract from the mid-mesocarp of 10-day postanthesis cantaloupe fruit were incubated with each of four fungal PGs. The starting level of activity, ~6 nkat (0.36 U), for each PG was constant throughout the various levels of added cantaloupe extract. The assay method is described in the text. Squares, *F. solani* PG; circles, *P. cucurbitae* PG; triangles, *A. niger* PG; diamonds, *D. bryoniae* PG.

where PG_0 = PG activity in the absence of added PGIP-containing extract, PG_{min} = residual reducing activity remaining at the maximal level of PGIP inhibition of PG, and PG_V = PG activity after treatment with a given volume of PGIP-containing extract.

The natural logarithm of $Fxr(PG_V)$ was plotted vs. the natural logarithm of $(V_f/(V_f - V))$ for each volume, V, of PGIP-containing extract added to a constant level of PG activity (Equation (1) of Fish and Madihally, 2004). V_f is the final volume of the solution containing PG, buffer, and PGIP. The factor, V_f, is a constant value since a compensating volume of buffer was added with each addition of a different volume of PGIP-containing extract. The points of such a plot yield a straight line for remaining fractional PG activities between ~0.1 and 1.0. The least squares linear equation fit to the data points was then used to solve for the volume of extract at a value of −0.693 for the ln $\{Fxr(PG_V)\}$, i.e. ln{0.5}. This value, together with the total units of PG activity maximally inhibited (the denominator term of Equation (1) above), was used to calculate units of PGIP activity per unit volume of extract (Fish and Madihally, 2004).

Statistical analyses

Statistical analyses, linear regression analysis, and mean and standard deviation determinations were performed with the aid of Statistica software, version 6 (Statsoft, Tulsa, OK).

Results

As illustrated in Figure 1, extracts of cantaloupe fruit inhibited the rate of reducing group formation from the polygalacturonate by crude extracts of *P. cucurbitae* and *F. solani* lesions and by commercial *A. niger* PG. No level of cantaloupe fruit extract produced an inhibition of PG activity from the *D. bryoniae* lesion extracts. This was true for all stages of fruit maturity and all tissues. That the observed loss of PG activity in the presence of cantaloupe fruit extracts was the result of PGIP activity and not protease inactivation of the enzyme was supported by four indirect observations. First, the presence or absence of serine protease inhibitor (AEBSF), metal chelator (EDTA), or pepstatin A had no effect on the degree of PG inhibition. Second, the presence or absence of bovine serum albumin in the pre-incubation and assay solutions had no effect on the degree of PG inhibition. Added serum albumin did help stabilize *P. cucurbitae* PG activity and so it was used routinely in all PG and PG-PGIP assays. Third, the inhibition of PG activity was almost immediate upon addition of cantaloupe tissue extract, and no further inhibition occurred thereafter. Lastly, the inability of cantaloupe fruit extracts to inhibit *D. bryoniae* PG under any circumstances also serves to rule out protease inactivation of the PG enzyme rather than PGIP activity as the cause of PG activity loss. A limited number of inhibition measurements with extracts of 10-day-old fruit vs. *P. cucurbitae* PG that had been purified by binding to Agarose-CmPGIP yielded inhibition curves similar to those with the crude extracts of *P. cucurbitae* lesions (data not shown).

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 the three cantaloupe

pathogens. The inhibitor was used at a level sufficient to achieve maximal inhibition of the enzyme in the reducing group-forming spectrophotometric assay. PG preparations of equal activities as determined by spectrophotometric assay were compared by viscometry. Extracts of 20-day postanthesis cantaloupe fruit mesocarp, added at a level to maximally inhibit reducing-group formation, slowed the rate of substrate viscosity decrease 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 unable to inhibit endo-PG from *D. bryoniae*. Heat denatured CmPGIP-containing extracts had no inhibitory effect on the endo-PG activity of any of the three extracts.

Figures 2 and 3 present the CmPGIP activity from various cantaloupe fruit tissues as a function of fruit maturity vs. *P. cucurbitae* (Figure 2) and *F. solani* (Figure 3) PGs, respectively. The 35 day postanthesis fruit were fully ripe and at full slip for the 2001 growing season; normally, 40 days postanthesis is considered to be the nominal age of ripe fruit. About one-fourth of the approximately 40 samples were re-extracted and re-assayed a second time to generate a composite estimate of the standard error of the mean. The composite standard error so estimated was $\pm 7\%$.

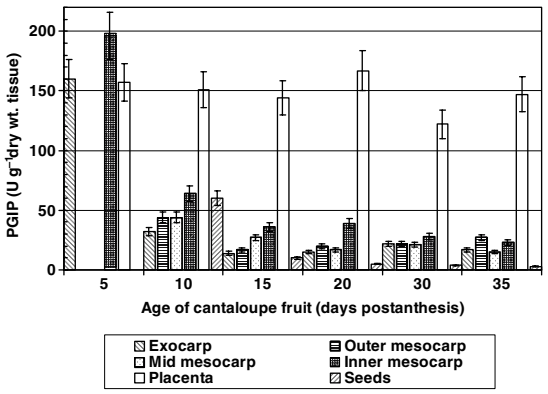

Figure 2. Cantaloupe fruit PGIP activity against *Phomopsis cucurbitae* PG. Fruit were harvested on the days postanthesis shown in the figure, and tissues were separated and processed as described under Materials and methods. PG and PGIP activities were determined as outlined in the text. The level of PG activity employed for each assay was ~6 nkat and was constant among a set of assays for a given tissue at a given time postanthesis. Depending on the level of PGIP activity in the tissue, the amount of tissue producing the extract employed for an assay ranged between 15 and 300 mg (see Materials and methods).

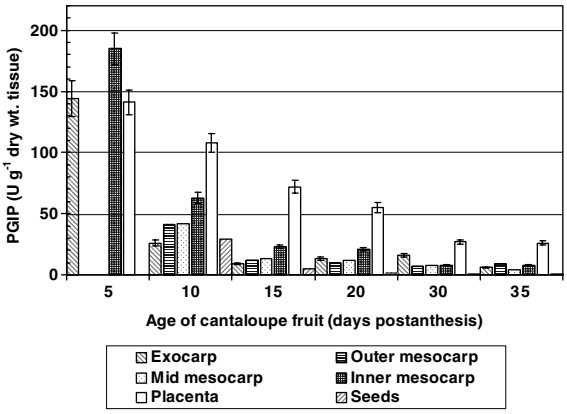

Figure 3. Cantaloupe fruit PGIP activity against *Fusarium solani* PG. Fruit were harvested on the days postanthesis shown in the figure, and tissues were separated and processed as described under Materials and methods. PG and PGIP levels were determined as outlined in the text. The level of PG activity employed for each assay was ~6 nkat and was constant among a set of assays for a given tissue at a given time postanthesis. Depending on the level of PGIP activity in the tissue, the amount of tissue producing the extract employed for an assay ranged between 15 and 300 mg (see Materials and methods).

As illustrated by Figures 2 and 3, the level of PGIP activity per unit weight of dried tissue (or, fresh tissue – not shown) is elevated in 5-day-old fruit. In most tissues, this high level decreased markedly between 5 and 10 days postanthesis prior to the onset of netting at about 12 days. This early high level of activity was approximately the same vs. both *P. cucurbitae* and *F. solani* PGs. No tissue at any stage of fruit development exhibited measurable levels of PGIP activity against *D. bryoniae* PG activity (data not shown). Assays of the female flower (ovary + petals + style-stigma) at anthesis and 1 day postanthesis from the 2002 crop, showed a level of PGIP of 320 units/g dry weight vs. both *P. cucurbitae* and *F. solani* PGs. The male flowers (petals + stamens) were not assayed. We can't conclude whether the PGIP would have been at this level at the corresponding flowering stages in the 2001 crop, but it is consistent with a very high level of inhibitor in the flower ovary and at the very early stages of fruit development. Fruit from the 2000 crop exhibited changes in PGIP levels consistent with those observed for the 2001 crop.

PGIP activity against *P. cucurbitae* PG remained at a high level in the seed cavity material (placenta) throughout fruit development. Conversely, PGIP activity against *F. solani* PG decreased as the fruit matured. Though this is most easily seen with the seed cavity tissue, a corresponding diminution in the ratio of PGIP activity vs. *F. solani* PG compared to PGIP activity vs. *P. cucurbitae* PG occurred for all other tissues. The ratio decreased from about 0.95 at 5 days postanthesis to about 0.25 at 35 days postanthesis (ripe). This same behavior occurred for tissues of fruit from the 2000 crop.

Units of PGIP activity were also normalized to the protein concentration of the extracts, and comparisons were made among tissues at the various stages of maturity of the fruit (data not shown). In general, results similar to those in Figures 2 and 3 were obtained. CmPGIP activity per mg protein vs. both *P. cucurbitae* and *F. solani* PGs was high in the 5–10 day old tissues, and the specific activity in placental tissue against *P. cucurbitae* PG remained high while that against *F. solani* PG decreased with fruit development. On the other hand, PGIP specific activity for exocarp, mesocarp, and seed tissues remained relatively constant after 10 days postanthesis. PGIP specific activity in seed after 10 days postanthesis was extremely small.

Discussion

The results quantify the distribution of CmPGIP activity among cantaloupe fruit tissues as a function of fruit development. The PGs utilized for this study were from natural fruit-rot pathogens of cantaloupe and were produced *in vivo* from the host–pathogen interaction. Limited studies with purified *P. cucurbitae* PG yielded results similar to those with its crude extract and support the validity of our measurements with the partially purified system. Also, *F. solani* has been shown to express only one endo-PG isoform when grown on cantaloupe fruit (Zhang et al., 1999); thus, the data with *F. solani* PG should represent the interaction of CmPGIP with only one isoform of the enzyme.

The overall levels of PGIP activity in developing cantaloupe fruit are consistent with what has been reported for fruit from other plant species. In these earlier studies, PGIP activity per unit weight of tissue was higher in the very immature stage than in the mature stage (Abu-Goukh et al., 1983; Johnston et al., 1993; Yao et al., 1999). This is the first investigation, however, in which PGIP activity has been quantified from different tissues within the fruit as it develops. Furthermore, this investigation demonstrates that there are differential levels of PGIP activity among fruit tissues (discussed in more detail below).

CmPGIP behaves like PGIPs from other plants in that it may be highly effective against some fungal PGs and totally ineffective against others. Since the same CmPGIP preparation at a constant concentration of PGIP was employed in Figure 1, the shapes of the curves give a qualitative comparison of the relative efficacies of CmPGIP against PGs from four different sources. It can be seen that CmPGIP's affinity is greatest for *F. solani* and *P. cucurbitae* PGs. Its binding to *A. niger* PG is somewhat weaker, although sufficient quantities will drive the inhibition to completion. In contrast, CmPGIP exhibits little or no inhibition of *D. bryoniae* PG at the highest levels of fruit extract tested. In the pure state, CmPGIP also exhibited little or no inhibition of *D. bryoniae* PG or *F. verticillioides* (formally *F. moniliforme*) PG (Fish and Davis, 2004).

In netted cantaloupe fruit, the apparent PGIP activity of most tissues is high from the flowering stage through the first week of fruit development. The exceptionally high level of PGIP activity measured in the female flower plus ovary, $\sim$320 U g^{-1} dry wt., is consistent with that measured for bean flower plus ovary (Salvi et al., 1990), while little PGIP activity was found in raspberry flowers (Johnston et al., 1993). Just prior to netting, the level of CmPGIP activity rapidly falls in most of the cantaloupe fruit tissues and then slowly diminishes during the remainder of the growth and ripening of the fruit. An interesting exception to this appears to be the seed cavity tissue that contains the apparatus for delivering nutrients to the developing seeds. The level of CmPGIP activity from this tissue measured against *P. cucurbitae* PG appears to change little over the 40 days of fruit development while its activity vs. *F. solani* PG progressively diminishes.

This differential change in the effectiveness of CmPGIP from seed cavity tissue against two different fungal sources of PG was observed for all the cantaloupe fruit tissues that were examined for

both the 2000 and 2001 crop years. The fact that this behavior was observed for fruit from two successive crop years demonstrates that the observation is not a one-crop artifact. The biological processes that result in this change in ratios of PGIP inhibition of *F. solani* and *P. cucurbitae* PGs cannot be definitively elucidated with the type of measurements conducted in this investigation. The assay we employed to quantify levels of PGIP activity yields only one variable, i.e. the amount of PG activity inhibited per unit volume of PGIP-containing extract added to the enzyme. Two physical parameters determine the inhibition, namely the quantity of inhibitor added and the affinity of its binding to the PG, and these cannot be individually extracted from the kinetic measurements for a single enzyme – inhibitor-protein system. By using two PG enzymes to separately assay the same PGIP-containing extract, it was hoped to be able to detect any putative qualitative changes that might occur in CmPGIP activity. The data obtained by such an approach suggest that a differential change occurred in the specificity of the PGIP(s) expressed by the fruit. The results also illustrate the potential gain in information to be realized from evaluating PGIP with PGs from a number of the plant's pathogens. Had PG from only a single pathogen source been employed in this investigation, the specificity changes in CmPGIP activity would not have been recognized. Thus, to minimize the possibility of drawing inaccurate conclusions based on a single enzyme – inhibitor-protein system, one is well advised to utilize multiple and varied pathogen systems for the enzyme sources.

Several scenarios can be offered to explain the observation that apparent changes occurred in the specificity of CmPGIP as the cantaloupe fruit developed. The possibilities include post-translational modification of CmPGIP that alters its enzyme affinity during fruit maturation, a product of fruit maturation that binds to CmPGIP and alters its binding properties, or multiple *pgip* gene products being expressed, each with different enzyme affinities and each with different rates of turnover or down-regulation. There is substantial evidence from several plant systems that *pgip* is a multi-gene family, that the gene products are nearly biochemically identical while being specificity-diverse, and that their expression can be both spatially and temporally regulated (DeLorenzo et al., 2001; DeLorenzo and Ferrari, 2002; D'Ovidio et al., 2004). Such regulation at the gene level certainly seems a likely scenario for cantaloupe fruit. A down-regulation of Cm*pgip* genes expressed in the early stages of fruit development, together with a concomitant up-regulation of Cm*pgip* genes expressed just before or at netting of the fruit, would be consistent with the differential change in CmPGIP specificity observed in this study. This scenario is also supported by treatment of data similar to these with an equilibrium binding model for enzyme – inhibitor-protein systems (Fish and Madihally, 2004). Data with 5- and 35-day cantaloupe fruit extracts and PGs from two different fungal pathogens treated with the above model are consistent with a form of PGIP produced in 5-day-old fruit that binds *P. cucurbitae* and *F. solani* PGs with nearly equal affinity. In 35-day-old fruit, only about 15% of this PGIP activity remains, and the other 85% of the PGIP activity binds *P. cucurbitae* PG with an affinity similar to the PGIP from 5-day-old fruit but binds/inhibits *F. solani* PG little or not at all (Fish and Madihally, 2004).

Bruton et al. (1998) have offered two hypotheses as to what is the important determinant in latent fungal infections. The first hypothesis is that a change in the molecular form of the fruit tissue cell walls that occurs with ripening presents the pathogen's enzymes with a higher concentration of appropriate substrate. The second hypothesis invokes the presence of specific inhibitors of cell-wall degrading enzymes that exist at sufficient concentrations in the immature fruit to curtail fungal colony growth and subsequent tissue maceration until the inhibitor level falls below a threshold level at-or-near the time of fruit maturity. With a nominal association constant between PG and PGIP of $\sim 10^9$ M^{-1} (DeLorenzo et al., 2001), inhibition can occur at very low concentrations of enzyme and inhibitor, and our inhibitory activity measurements were not sensitive enough to detect such a putative threshold PGIP concentration. Certainly, a diminution in the efficacy of the PGIP isoform being expressed as well as its expression occurring at diminished levels could provide the conditions for crossing a putative latency threshold.

It is interesting to contemplate why a plant's vegetative tissues increase in PGIP activity with age of the seedling (Salvi et al., 1990) while a majority of its fruit tissues decrease in PGIP

activity with age (Abu-Goukh et al., 1983; Johnson et al., 1993; Yao et al., 1999; this study). For the limited cases investigated, only the reproductive apparatuses are observed to possess and maintain high levels of PGIP activity. The ovary, style-stigma, and stamen of the bean flower each possess high levels of PGIP activity (Salvi et al., 1990), as does the cantaloupe ovary + petals + style-stigma (this study). The results of this study suggest that the elevated level of PGIP activity observed in the immature ovary is selectively maintained in that tissue for facilitating and maintaining seed growth and development. Thus, it may be that except for the initial stages of fruit development, the primary function of CmPGIP in fruit is protection of the seed-nurturing apparatus rather than protection of the whole fruit.

Acknowledgement

The author would like to thank Rick Houser for providing expert technical support.

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