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Purification and Characterization of an endo-Polygalacturonase from the Gut of West Indies Sugarcane Rootstalk Borer Weevil (*Diaprepes abbreviatus* L.) Larvae*

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ABSTRACT. An endo-polygalacturonase (PG) (EC:3.2.1.15) with a pI of 9.4 and an M_r of 44,500 was purified to electrophoretic homogeneity from the gut of West Indies sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* L.) larvae. Hydrolytic activity was maximal in 150 mM sodium acetate, pH 5.5, at 30°C. Kinetic determinations yielded an apparent K_m of 3.68 mg polygalacturonic acid (PGA)/ml and a V_{max} of 283 μmol galacturonic acid/min/mg protein for PGA. Enzymatic activity was inhibited by a polygalacturonase inhibitor protein from "Hamlin" orange flavedo. The purified protein does not appear to be glycosylated, and its N-terminal sequence showed no homology to any PG protein sequences in data banks. COMP BIOCHEM PHYSIOL 118B;4:861–867, 1997.

KEY WORDS. Citrus, herbivory, insect, N-terminal sequence, polygalacturonase inhibiting protein, PGIP, plant defense

INTRODUCTION

The West Indies sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* L.) is a pest of economic importance in the Caribbean basin. The weevil has a wide host range, but in Florida, *Citrus* spp. appears to be the preferred host (27). The larval stage of the weevil is subterranean and causes great damage by feeding on the bark of roots and causes plant death by girdling the tree at the crown of the roots. Losses estimated at more than $75 million are suffered by Florida citrus growers every year due to cost of control, resetting trees and reduced yield (12).

Strategies for controlling and limiting the spread of the weevil include quarantine of nursery plant material, use of pesticides and the introduction of entomopathogenic nematodes (28). Although these measures have provided excellent protection in nursery situations, they are not adequate in the field, and new more efficacious approaches are needed.

Insects and pathogens that attack plants use an array of polysaccharide hydrolyzing enzymes for degradation of the plant material (e.g., endo-polygalacturonase [PG]). PG hydrolyzes the α-1,4 glycosidic bonds of non-esterified portions of pectic substrates, which are major components of plant cell walls. Plants also produce PGs that are involved in pollination (26,34) and fruit ripening (5,22). Plant and fungal PGs have been studied extensively and many have been purified. Although the presence of PG has been reported in both sucking (2,16) and chewing (32) insects, little is known about its nature. To date, only one report has described the purification of an insect PG (32), but no sequence data has been published.

Here, we describe the purification, characterization and N-terminal amino acid sequence of PG from larvae of the West Indies sugarcane rootstalk borer weevil.

MATERIALS AND METHODS

Insect Rearing

Weevil larvae were from a laboratory colony reared on an artificial diet (4). Ten-week-old larvae weighing 0.5–0.8 g each were used for experiments.

Protein Determination

Protein concentrations were determined using two methods (7,15). Bovine serum albumin was used as the standard in both assays.

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Protein Purification

Guts (135 g) dissected from 2500 10-week-old larvae were placed immediately in liquid nitrogen and stored at −80°C until used. Dissected guts were added to 500 ml of 0.1 M sodium phosphate (pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 20 mM 1,4-dithiothreitol) and homogenized on ice for 2 min with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) fitted with a PTG 36-50 probe. The crude homogenate was centrifuged at 20,000 ***g*** at 4°C for 20 min, and the resulting supernatant was passed through two layers of Miracloth (CalBiochem, La Jolla, CA). Ammonium sulfate was added gradually to the supernatant to 80% saturation and left stirring for 2.5 hr at 4°C. The salt solution was centrifuged at 20,000 ***g*** at 4°C for 20 min; the resulting protein pellet was resuspended in 20 mM Tris (pH 8). The solution was recentrifuged and the supernatant (hereafter designated AS) collected.

AS was equilibrated and concentrated in 20 mM Tris (pH 8; buffer A) using an ultrafiltration cell (Amicon, Beverly, MA) fitted with a PM10 filter and then loaded onto an anion exchange column (Q-Sepharose, Pharmacia, Piscataway, NJ). The column was washed with buffer A to elute unbound proteins. The column-bound proteins were eluted in a linear gradient of 0–0.5 M NaCl in buffer A. The void fraction (QS-void) contained all PG activity. The QS-void was concentrated as described above and loaded in three batches at 0.5 ml/min onto an affinity column packed with 20 g of cross-linked apple pectate (25). The column was washed with buffer A and the column-bound proteins were eluted with 0.5 M NaCl in buffer A. Only the column-bound protein fraction contained PG activity. The buffer of this fraction was exchanged with 20 mM Bicine (pH 8; buffer B) and concentrated as above. The concentrated sample was loaded onto a cation exchange column (Mono S, Pharmacia) and washed with buffer B to elute unbound proteins. The column-bound proteins were eluted in 2-ml fractions using a linear gradient of 0–1 M NaCl in buffer B and assayed for PG activity. Fractions containing PG activity were pooled (Mono-S).

Enzyme Assays

Polygalacturonase activity was measured by incubating enzyme samples in 100 μl of 150 mM sodium acetate (pH 5.5) at 30°C unless specified otherwise. The reaction was initiated by the addition of 100 μl of preheated 0.2% (w/v) polygalacturonic acid (PGA) (potassium salt, from oranges; Sigma, St. Louis, MO) dissolved in the above buffer. After incubating for 15 min, the level of hexuronic acid-reducing sugars released was measured (21). Galacturonic acid (GA) was used as the standard. PG activity was expressed as μmol GA equivalents/min/mg protein.

Thermal stability of PG was measured by preincubating it at different temperatures for 10 min and then running the reaction at 30°C.

Pectate lyase activity was determined (11) by measuring a change in absorbance at 232 nm using 0.24% (w/v) PGA as the substrate in 60 mM Tris (pH 8.5) containing 0.6 mM $CaCl_2$.

Pectin lyase activity was measured as for pectate lyase except that apple pectin (Sigma) was used as the substrate.

α-Amylase activity was measured by diluting an appropriate amount of enzyme in a total volume of 125 μl of buffer (20 mM sodium phosphate, pH 6.9, 6 mM sodium chloride) and equilibrating the mixture at 25°C. Prewarmed potato starch (1% w/v; Sigma) in buffer (125 μl) was added to initiate the reaction. The reaction was terminated after 3–5 min, and the level of reducing sugars released was quantified (33) using glucose as the standard. α-Amylase activity was expressed as μmol glucose/min/mg protein.

Cellulase activity was measured by incubating enzyme samples with 0.2% (w/v) carboxymethyl-cellulose (sodium salt, medium viscosity; Sigma) in 50 mM sodium acetate (pH 5), for 15 min at 37°C. Reducing sugar released was quantified (33) using glucose as the standard. Cellulase activity was expressed as μmol glucose/min/mg protein.

Lysozyme activity was measured, using *Micrococcus lysodeikitcus* (Sigma) as substrate (24). Lysozyme activity was expressed as ΔA_{510}/min/mg protein.

N-Terminal Sequencing

Purified protein was fixed onto polyvinylidene difluoride membranes using ProSpin columns (Applied Biosystems, Foster City, CA) following manufacturer's instructions. N-Terminal sequencing was performed by the Interdisciplinary Center for Biotechnology, University of Florida (Gainesville, FL).

Capillary Electrophoresis

The purity and pI of the purified PG was determined by capillary electrophoresis (CE) using a Spectraphoresis 1000 (Thermal Separations, Fremont, CA). Protein samples were resolved using a 70-cm-long capillary with a 50-μm internal diameter. The samples were electrophoresed in 40 mM sodium tetraborate buffer of varying pH at 25°C and 15 kV with an average current of 30 mA and resistance of 0.5 gΩ. Benzyl alcohol was used as the neutral marker. The pI was determined as the pH in which the protein co-migrated with the neutral marker (18).

PG Digestion Pattern

Purified PG (11.5 μg of protein was incubated with 44 μg PGA in 400 μl of 150 mM sodium acetate (pH 5.5) at 30°C. At various time intervals, aliquots (10 μl) of the reaction mixture were spotted onto 0.25-mm silica gel GHL plates (Analtech, Newark, DE) that had been preconditioned (0.1 M sodium phosphate buffer, pH 7.4) and heated at 100°C

TABLE 1. Summary of steps in the purification of gut polygalacturonase from *Diaprepes abbreviatus* larvae

Purification step	Total protein (mg)	Specific activity (μmol GA/min/mg prot)	Total activity (μmol GA/min)	Fold purification*
Crude homogenate	2529.6	0.5	1214.2	1.0
Centrifuged crude	1734.5	0.6	1127.4	1.3
AS	11,331.1	0.3	413.3†	0.6
Q Sepharose FF void	349.8	0.4	1273.0	0.8
Affinity column	27.0	21.9	592.5	45.7
Mono S fraction	3.9	61.3	238.0	127.5

*Based on specific activity.
†Low activity possibly due to high salt concentration.

for 1 hr. The plates were developed using ethyl acetate–acetic acid–water (2:1:1). Carbohydrates were visualized (6). The rate of reduction in viscosity of a PGA solution by the purified PG was determined (3). PG (20 μg) was incubated at 30°C with a solution of 7% (w/v) PGA in 150 mM sodium acetate (pH 5.5). The change in viscosity was measured as the increase in the flow rate of the solution through a 1-ml glass pipette.

Inhibition by Polygalacturonase Inhibitor Protein

Purified PG (11.5 μg of protein) was preincubated at room temperature for 15 min with varying concentrations of a semi-purified PGIP from "Hamlin" orange flavedo. The mixture was placed in a water bath at 30°C and the reaction initiated by the addition of PGA. The reaction was terminated after 15 min. Inhibition was expressed as percent reduction in PG activity compared with control samples. The digestion patterns of PGA by the purified PG in the presence and absence of citrus PGIP was characterized by TLC as described above.

Polyacrylamide Gel Electrophoresis

Protein samples were resolved by SDS-PAGE (14) using a Bio Rad (Hercules, CA) Mini-Protean II apparatus. After electrophoresis, proteins were either visualized by staining with Coomassie Blue or blotted onto nitrocellulose (0.2 μm, Bio Rad) filters using a semi-dry transfer cell according to manufacturer's instructions (Bio Rad). Polyclonal antibodies raised to the purified protein in rabbits (BAbCo, Richmond, CA) were used for immunodetection.

Glycosylation Determination

The purified PG was assayed for the presence of glycosides using a BioRad Immun-Blot Kit for glycoprotein detection. One microgram of PG was resolved by SDS-PAGE and subsequently blotted onto nitrocellulose filter. The filter was treated and developed as per manufacturer's instructions. Ovalbumin was used as a positive control.

Bacterial Isolation from Weevil Larval Gut

We investigated the presence of microorganisms in the weevil gut to determine the origin of the PG. Thirty weevil larvae (10 weeks old) were dipped in 70% (v/v) ethanol–water to remove extradermal bacteria. The ethanol was evaporated, and subsequently the guts of the larvae were removed and placed on LB-agar plates supplemented with 10% (w/v) weevil feed and 5% (w/v) apple pectin and incubated under aerobic conditions. Only one plate showed growth of bacterial colonies. These colonies were isolated and cultured in LB broth containing 5% apple PG. Total protein extracts from the cultured bacteria were used to measure PG activity.

RESULTS

Protein Purification and Physical Characterization

Crude protein extracts from larval guts were assayed for PG and found to have activities of ~0.48 μmol GA/min/mg protein. A single PG was purified 128-fold based on specific activity from the gut of the weevil larvae (Table 1). The protein appeared as a single band with an apparent M_r of 44,500 on SDS-PAGE (Fig. 1A, lane 7).

Immunodetection of total protein extracts from the larval guts with the polyclonal antibody raised to the purified larval gut PG showed a single band in the extract (Fig. 1B). The protein also appeared as a single peak when resolved by CE and comigrated with the neutral marker at a pH of 9.4 indicating the pI of the protein (Fig. 2). The pI value 9.4 was corroborated by isoelectric focusing-PAGE using a gradient gel (data not shown). The protein showed no glycosylation when assayed for total carbohydrates using the BioRad glycosylation detection kit (data not shown).

N-Terminal Sequencing

The N-terminal sequence of the protein is shown in Table 2. The sequence showed no homology to any PG sequence published in data banks.

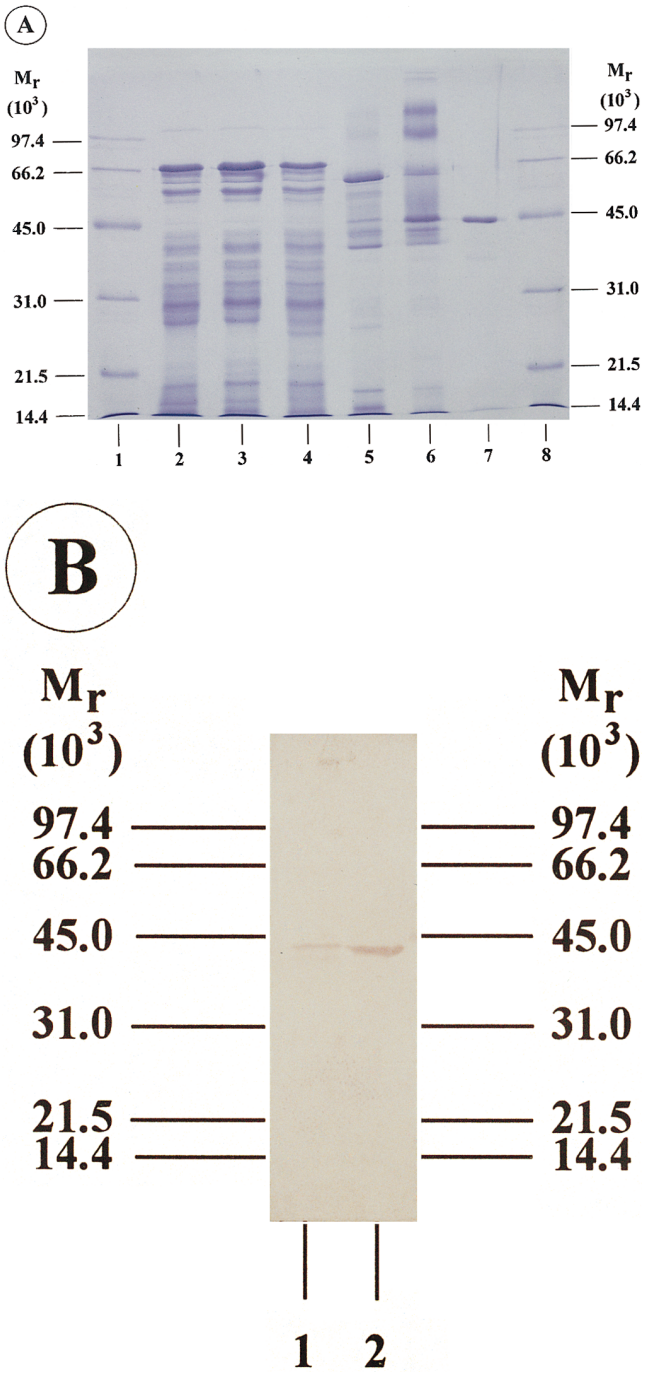

FIG. 1. (A) SDS-PAGE (12% w/v) of the various protein fractions obtained during purification of gut PG from larvae of *Diaprepes abbreviatus*. Lanes 1 and 8, molecular weight markers; 2, crude weevil gut protein (10 μg); 3, 20,000 *g* supernatant crude gut protein (10 μg); 4, ammonium sulfate precipitated fraction (10 μg); 5, void fraction from the Q-Seph FF anion exchange column (5 μg); 6, protein fraction bound to pectin affinity chromatography column (5 μg); 7, purified larval guts PG eluted from MonoS cation exchange chromatography column (1 μg). (B) Western blot of weevil protein immunostained using polyclonal antibody raised to the purified weevil PG. Left column indicates M_r (×1000); lane 1, total protein extracted from larval guts (10 μg); lane 2, purified PG from larval guts (1 μg).

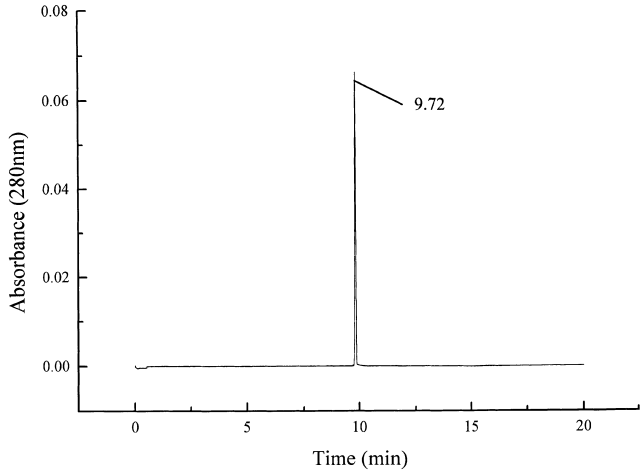

FIG. 2. Electropherogram (E-gram) of purified *Diaprepes abbreviatus* larval gut PG protein. This E-gram represents the protein resolved in a 75-cm column at 25°C and 15 kV with an average current of 30 mA and resistance of 0.5 gΩ, in a 40 mM sodium tetraborate buffer, pH 9.4.

Enzyme Characteristics

The purified protein was assayed for PG, pectate lyase, pectin lyase, lysozyme, cellulase and α-amylase activities; however, only PG activity was observed. PG activity was maximal in 100–200 mM sodium acetate buffer at pH 5.5–6; activity decreased sharply below or above these molar concentrations and pH ranges. Maximal activity was observed at 55°C; however, stability experiments indicated significant loss of activity above 50°C and total deactivation at 70°C. Enzyme activity in MES buffer, pH 5.5, was lower than in acetate buffer, but its activity was less affected by higher buffer concentrations (Fig. 3). At optimal conditions (150 mM sodium acetate, pH 5.5 and 30°C) using PGA as the substrate, the apparent K_m and V_{max} were calculated to be 3.68 ± 0.84 mg PGA/ml and 283 ± 54 μmol GA/min/mg protein, respectively.

The mode of action of the purified PG was determined using viscometric and thin-layer chromatography (TLC) methods. The viscosity of a 7% (w/v) PGA solution was reduced by 70% within 15 min of incubation, indicating that the weevil PG is an endo-PG. An exo-PG would, presumably, take longer to have this amount of reduction in viscosity. Visualization of PGA digestion by TLC (Fig. 4A) further confirmed that the enzyme is an endo-PG, producing a wide range of oligogalacturonides, with production of a small amount of galacturonic acid after long incubation times (Fig. 4A, lanes 7 and 8).

Inhibition by a Citrus Polygalacturonase Inhibitor Protein (PGIP)

Purified PG was inhibited in a concentration-dependent manner by a semi-purified preparation of PGIP obtained

TABLE 2. N-terminal comparison of PG sequences from different sources

PG source		Amino acid								
Diaprepes abbreviatus		I	Y	T	Y	V	I	G	R*	G
Tomato (*Ailsa craig*)†	2A	M	V	I	Q	R	N	S	I	L
Tobacco‡		V	L	L	F	L	A	H	F	G
Aspergillus flavus§	Pec A	M	Q	L	L	Q	S	S	V	I
	Pec B	M	H	F	Q	L	L	V	L	A
Aspergillus niger‖		M	H	S	Y	Q	L	L	G	L

*Indicates low confidence in the amino acid.
†Bird *et al.* (5).
‡Tebbutt *et al.* (34).
§Whitehead *et al.* (35).
‖Bussink *et al.* (8).

from "Hamlin" orange flaved (Fig. 5). The I_{50} for this PGIP preparation using 28.7 μg/ml of PG was ~77.5 μg flavedo protein/ml. PG produced only high-molecular-weight oligogalacturonides in the presence of PGIP (Fig.4B), whereas in the absence of inhibitor smaller oligogalacturonides were produced.

DISCUSSION

PGs have been reported in a number of insects (20), where it is used for stylet penetration into plant cells by aphids (19) and by leaf-chewing insects to break down ingested plant materials (32). Little information is available regarding the nature of these enzymes in insects. Although the 44,500 M_r of the *D. abbreviatus* PG reported here is higher, its other characteristics are not drastically different from a PG purified from rice weevil, *Sitophilus oryzae* (32). The *S. oryzae* PG is also an endo-PG with an M_r of 38,000, a K_m of 1.1 (mg/ml) and a pH optimum of 5.5. PG activity in *Lygus* spp. is also reported to be optimal between pH 5 and 6 (2).

The method used for the isolation of microorganisms from the weevil gut was not exhaustive and the presence of symbiotic bacteria cannot be discounted (9), the lack of sequence homology of the purified protein with any of the published microbial and plant PG sequences, coupled with the lack of PG activity in bacteria isolated from the weevil gut, suggests that the protein isolated here is of insect origin.

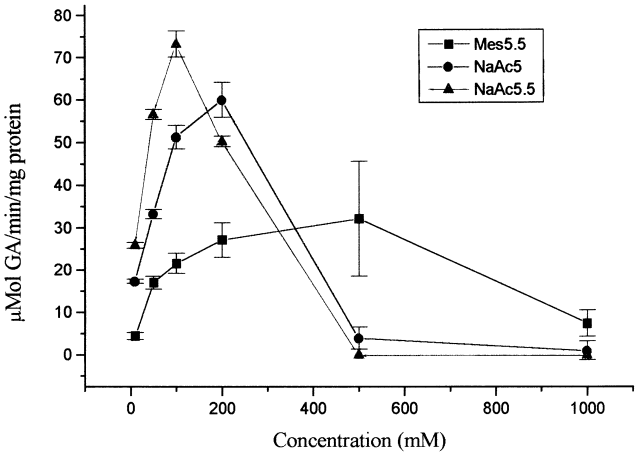

FIG. 3. Effects of buffer composition and concentration on *Diaprepes abbreviatus* larval PG activity. PG activity is mean ± SE for five reactions.

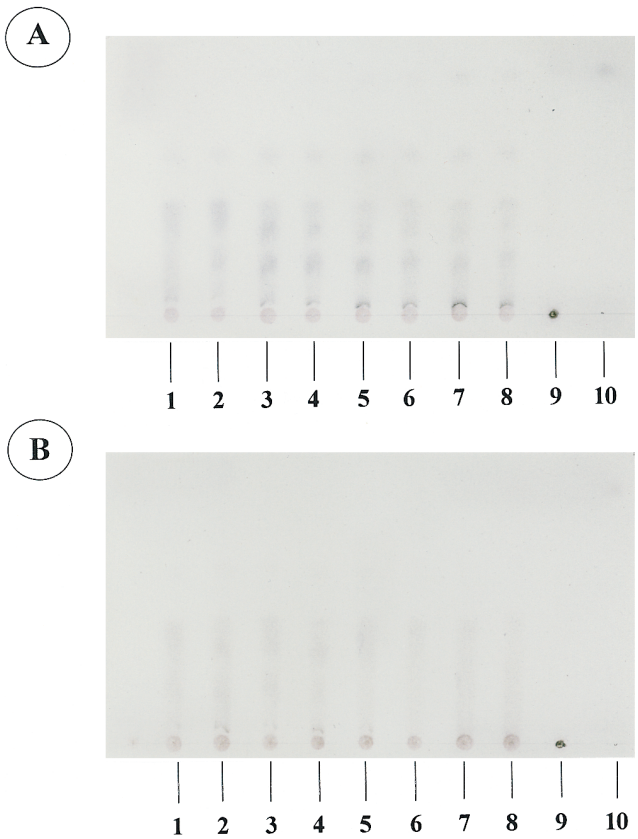

FIG. 4. TLC visualized digestion profile of polygalacturonic acid by (A) the purified protein (11.5 μg) and (B) purified protein (11.5 μg) preincubated (15 min at 25°C) with the semi-purified PGIP (60 μg) from flavedo of "Hamlin" oranges. Samples were taken at various time intervals after the addition of the substrate. Lanes 1, 1 min; 2, 5 min; 3, 10 min; 4, 20 min; 5, 40 min; 6, 60 min; 7, 90 min; 8, 120 min; 9, substrate control; 10, GA control.

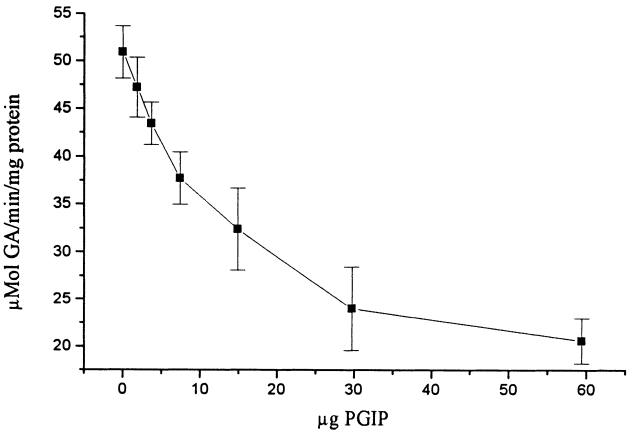

FIG. 5. PGIP inhibition of *Diaprepes abbreviatus* polygalacturonase. Purified *D. abbreviatus* larval PG (11.5 µg) was preincubated at 25°C for 15 min with various concentrations of semi-purified orange flavedo PGIP before the addition of the substrate. PG activity is mean ± SE for five reactions.

Although the ability of various plant PGIPs to inhibit fungal and bacterial PGs has been well documented (13) and has been correlated with plant resistance to pathogen infection (1,23), no data have been reported on the interaction of plant PGIPs with insect PGs. Here we have shown that PG isolated from *D. abbreviatus* gut is inhibited by a citrus PGIP; however, we do not know what the effects of the inhibition would be *in vivo*. It is interesting that the I_{50} of the citrus PGIP for PG from *D. abbreviatus* is about the same as that obtained for PG from *Aspergillus niger* (data not shown). Our data indicate that the citrus PGIP inhibits the production of small chain oligogalacturonides by insect PG without affecting the production of the larger oligogalacturonides (i.e., larger than 10 GA units). This pattern of inhibition has also been reported for fungal PG and it has been suggested that such action increases the induction of plant defense mechanisms by increasing the amount of oligogalacturonides in the 10–20 GA range (10,31); oligogalacturonides of this size are reported to be elicitors of defense responses.

Inhibitory proteins like PGIP and pathogenesis-related proteins may play roles in defending plants against arthropod pests. The susceptibility of citrus rootstocks to damage by larval root weevil feeding varies greatly with cultivar (30). We have previously reported that larval feeding induces chitinases in the roots of some of the citrus cultivars exhibiting weevil resistance and demonstrated that these chitinases can digest peritrophic membranes of *D. abbreviatus* larvae (17).

It is possible that other proteins are also induced in resistant citrus cultivars that inhibit digestive enzymes in the weevil gut. Researchers working with the cowpea weevil (*Callosobruchus maculatus*) and Azuki-bean weevil (*C. chinensis*) larvae have shown that transgenic peas expressing the gene for α-amylase inhibitor from wheat are less susceptible to larval infestation (29) than non-transformed plants. Therefore, inhibition of digestive enzymes of *D. abbreviatus* might provide an effective pest management strategy.

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