INHIBITION OF AN EXTRACELLULAR POLYGALACTURONASE FROM *GEOTRICHUM CANDIDUM* BY A PROTEINACEOUS INHIBITOR ISOLATED FROM LEMON FRUITS

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ABSTRACT

An extracellular polygalacturonase [EC 3.2.1.15], named PG-543, was purified from the culture of *Geotrichum candidum* IEV 543, a phytopathogenic fungi isolated from infected lemon. The molecular mass of the enzyme was estimated to be 26 kDa by SDS-PAGE. The pH optimum determined was 5.5; the enzyme showed high stability in the pH range between 4.0 and 6.0. The temperature optimum was 37 ºC. Furthermore, a proteinaceous inhibitor of the polygalacturonase (PG) named PGIP was isolated from lemon albedo. This protein was able to maintain the inhibitory activity in a wide range of pH and temperature. The maximal interaction between PG-PGIP was reached after 20 min of contact. PGIP did not show toxic effect on *Artemia salina* in the concentration range that was active on PG enzyme. Also, the proteinaceous inhibitor did not show mutagenic nor phytotoxic effects.

Our results suggest that, the PGIP isolated from lemon could be used as a natural product to decrease the propagation of pathogenic fungi responsible for postharvest diseases in citrus by the inhibition of hydrolytic enzymes secreted by pathogens.

Keywords: polygalacturonase (PG); *Geotrichum candidum*; PG-inhibitor protein; lemon fruits; PG-PGIP interaction

INTRODUCTION

Polygalacturonases (PGs) catalyze the hydrolysis of 1,4-α-D-galactoside-uronic linkages of plant cell wall pectin. PGs are widely distributed among fungi, bacteria and many types of yeasts and also found in higher plants and some plant parasitic nematodes (**Kazuo *et al.*, 2002.; Contreras Esquivel and Voget, 2004; Niture and Pant, 2004; Jacob *et al.*, 2008**). The PGs from microbial sources have been characterized and classified into the glycoside hydrolase family 28 (http://afmb.cnrs-mrs.fr/CAZY/index.html). They are important agents of fungal pathogenicity necessary to colonize the plant tissue during their invasion. These enzymes decompose plant cell wall components causing a localized breach which constitutes the gate of infection (**Lazniewska *et al.*, 2012**). PGs enable a pathogen to invade plant tissues, and also, their activity may trigger plant defense responses. Plants possess numerous enzymes inhibitors, such as polygalacturonase inhibitor proteins (PGIPs) which may protect them from destructive effects of fungal hydrolases (**Lagaert *et al.*, 2009; Protsenko *et al.*, 2010; Schacht *et al.*, 2011**). Besides PGIPs, there are inhibitors of pectin lyases, pectin methylesterases, pectate lyases, endoxylanases, xyloglucan-specific endoglucanases, whose activities, functions and distribution among plant species have been described (**Favaron *et al.*, 1994; Isla *et al.*, 2002; Faize *et al.*, 2003; Lagaert *et al.*, 2009**).

Geotrichum candidum Link: Fr. is a yeast-like fungus that causes diseases in humans, animals and plants (**Carmichael, 1957; Butler, 1960; Butler *et al.*, 1965; Suprapta *et al.*, 1996**). This fungus has been reported from many citrus growing areas of the world (**Brown and Eckert, 1988; Hershenhorn *et al.*, 1992**). It causes sour rot in citrus fruits, which is an important cause of postharvest disease and economic losses (**Nakamura *et al.*, 2008**). The fungus penetrates the fruit only through injuries, particularly deep injuries that extend into the albedo. These injuries may be caused by insects or mechanical means, such as thorn or stem punctures, or by plugging at harvest. This disease is one of the main causes of postharvest losses of citrus in Tucumán province (Argentina), which is a leading citrus producing region in the world; particularly of lemon and lemon industrial products such as concentrated lemon juice, lemon essential oil and dried lemon peel. The destinations for fresh fruit are the European Union, Russia, Ukraine, Japan, Canada, Hong Kong, Southeast Asia, and Asia Minor and for industrialized products, United States, Canada, European Union, Japan, Australia and Israel (Asociación Tucumana de Citrus, 2013). Synthetic

fungicides have a major role in order to reduce postharvest losses due to phytopathogenic fungi. However, problems resulting from their extensive use, as well as their associated health and environmental risks, have promoted the search for new and safer alternatives. Natural products capable of inhibiting fungi hydrolytic enzymes could be important tools in preventing these kinds of infections. In the present work we report the purification and biochemical characterization of a PG from a citrus pathogen *G. candidum* strain. Also, the effect of a glycosidase inhibitory protein isolated from lemon fruits on the purified PG was evaluated.

MATERIALS AND METHODS

Reagents

All reagents used were analytical grade and purchase from Merck & Co. or Sigma Aldrich Argentina.

Production, purification and characterization of polygalacturonase enzyme from *Geotrichum candidum*

Microorganism

The *G. candidum* strain (IEV 543) was isolated from infected citrus fruits in order to support their pathogenicity and then identified by the Laboratory of Mycology from Facultad de Bioquímica, Química y Farmacia (UNT). This isolate was routinely cultured on potato dextrose agar (PDA, Merck) slants for preservation.

Enzyme production

The enzyme was prepared according to **Torres *et al.* (2011)** with some modifications. *G. candidum* was grown on a pectin containing medium (peptone, 10 g/L and pectin, 30 g/L), without shaking, at 28 °C for 10 days. Cell-free supernatants were obtained by centrifuging the cultures at $21,000 \times g$ for 15 min (4 °C). Supernatants (protein: 3.15 g/mL) were concentrated by precipitation with 100% solid $(NH_4)_2SO_4$, dialyzed against 20 mM sodium acetate buffer pH 5.5 and then used as a source of PG activity.

Enzyme purification

The crude extracts were precipitated with 100% solid $(NH_4)_2SO_4$ and concentrated six fold by ultrafiltration using an Amicon system with a membrane with a cutoff point of 10 kDa. The concentrated enzyme was applied to a DEAE-Sepharose CL 6B (10 x 1.5 cm) column equilibrated with sodium acetate buffer (0.2 mM, pH 5.5) and eluted at a flow rate of 0.5 mL/min with a linear gradient of NaCl (0–1 M). The fractions containing polygalacturonase activity were pooled and applied to a Sephadex G-150 column (20 x 2 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.5 and eluted with the same buffer. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. The fractions showing maximum polygalacturonase activity were pooled for further studies.

Enzyme assay

The PG activity was determined by measuring the release of reducing sugars, according to the Nelson–Somogyi method (Nelson, 1944; Somogyi, 1945) using sodium polygalacturonate as substrate. The reaction mixture for standard assay contained 20 µL of enzyme preparation in 0.2 M sodium acetate buffer pH 5.5 and 0.2% (w/v) sodium polygalacturonate in a final volume of 100 µL. Enzyme reactions were started by the addition of the substrate to the reaction mixture and incubated at 37 °C at different time (10 to 180 min). The reaction was stopped by boiling the reaction mixture. One enzyme unit (EU) activity was defined as the amount of enzyme which produced 1 µmole of reducing sugars per min at pH 5.5 and 37 °C.

Determination of protein concentration

Protein quantification was carried out by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Analytical electrophoresis

SDS-PAGE (15%) was performed in order to check the homogeneity and the molecular mass of the enzyme (Laemmli, 1970). Protein bands were visualized by $AgNO_3$ staining technique.

Molecular mass estimation

The molecular weight of purified PG was estimated by SDS-PAGE (Laemmli, 1970). SDS-denatured molecular weight markers were used for the construction of the calibration curve.

Substrate specificity and mode of action of the PG

Sodium polygalacturonate and citric pectin (0.2% w/v) were used as substrate in order to evaluate the substrate specificity of the enzyme. Solutions containing reaction products were filtered through Amberlite MB-3 and concentrated by evaporation for further evaluation by TLC. The samples were spotted onto a silica gel TLC plates (Kieselgel 60 0.2mm, Merck). The plates were developed in ascending direction with butanol:pyridine:water (6:4:3 v/v) as mobile phase and revealed with diphenylamine/aniline/phosphoric acid reactive (Chaplin and Kennedy, 1986). Products containing uronic acid residues appeared as blue spots. The reaction products were also evaluated at 235 nm in order to identified lyase activity. Endo-polygalacturonase activity of PG-543 was confirmed by measuring the enzyme activity using the Ruthenium red method according to **Torres *et al.* (2011)**.

Effect of pH and temperature on enzyme activity and stability

The influence of pH on polygalacturonase activity was measured by performing the activity assay at pH values ranging from 3.0 to 10.0 at a constant temperature of 37 °C. The optimum pH of the enzyme was investigated in 40 mM citrate-phosphate (pH range 3–4), sodium acetate (pH 5.5), sodium phosphate (pH range 7–8) and glycine-NaOH (pH 10) buffers. Optimum temperature for enzyme activity was determined by varying the assay temperature from 25 to 60 °C at the optimum pH. The pH stability of the enzyme was determined by pre-incubating the enzyme in the different aforementioned buffers at the optimum temperature and measuring the residual activity after 45 min. Temperature stability was studied by incubating the enzyme at different temperatures (25–60 °C) at the optimum pH for 45 min and measuring the residual activity.

Enzyme kinetics

Kinetic constants, Km and Vmax, of the enzyme were calculated by fitting activity data with different substrate concentrations (2–30 mg/mL) to a linear regression on Lineweaver-Burk double-reciprocal plot. The enzyme concentration was kept constant.

Isolation and characterization of inhibitory protein of PG from lemon albedo

Extraction and partial purification of inhibitory protein of PG from G. candidum

Lemon fruits were washed with distilled water and peeled. Flavedo and albedo were excised and cut into small segments (approximately 1 - 5 mm²). A sample (100 g) of albedo was homogenized with the aid of a high-speed blender, in 200 mL of sodium acetate buffer (20 mM; pH 5.5) containing NaCl (1 M) and β-mercaptoethanol (1 mM). This suspension was stirred overnight at 4 °C. Then, in order to separate the solid components, the slurry was filtered and centrifuged at 10,000x*g* for 15 min. The supernatant was carried out to 100% saturation with solid ammonium sulphate and centrifuged at 10,000x*g* for 15 min. The insoluble fraction was recovered, dialyzed against the same buffer without NaCl (3 h) and re-suspended in the same buffer. After that, the protein extract was treated with PVPP (10%) to obtain a polyphenolic-free proteinic extract. The protein fraction was applied to a DEAE-Sepharose CL 6B (10 x 1.5 cm) column equilibrated with sodium acetate buffer (200 mM, pH 5.5) and eluted at a flow rate of 0.5 mL/min with a linear gradient of NaCl (0–1 M) in the same buffer. The eluted fractions were monitored at 280 nm for protein determination and assayed for PG inhibitory activity. The fractions showing inhibitory activity were pooled for further studies. SDS-PAGE was performed as previously stated in order to check the homogeneity of the inhibitory protein.

Effect of inhibitory protein on PG

Effect of the concentration of inhibitory protein on PG activity

The PG activity was measured in a mixture containing different inhibitor concentration (0.1-1.2 µg/mL), enzyme (1.15 EU) and sodium polygalacturonate (2-30 mg/ml) as substrate in 0.2 mM sodium acetate buffer pH 5.5. The reaction mixture was maintained at 37 °C during 30 min. The PG activity was determined by measuring the release of reducing sugars, according to the Nelson–Somogyi method (**Nelson, 1944; Somogyi, 1945**). The inhibition constant, Ki, was determined by Dixon plot.

Effect of contact time between enzyme and inhibitor on PG activity

The effect of contact time between PG and inhibitor was determined. The enzyme (1.15 EU) and inhibitory protein (0.9 µg/mL) were pre-incubated at pH 5.5 during different times (5 to 40 min). Then, the substrate (0.7 mg/ml) was added to the reaction mixture and the activity was measured as previously stated.

Effect of pH on inhibitory protein stability

The inhibitor was pre-incubated at room temperature in solutions with different pH (between 2 and 10) during 45 min. Then, the activity of inhibitory protein on PG was assayed at pH 5.5.

Effect of temperature on protein inhibitory stability

The inhibitor was preincubated at different temperatures (between 25 °C and 60 °C) for 45 min. Then, the activity of inhibitory protein against PG was assayed at pH 5.5.

Determination of protease and glycosidase activities in inhibitory protein fraction

Protease assay

Protease activity was assayed by using azocasein (Sigma-Aldrich) as substrate. The reaction mixture for standard assay contained 10 µL of PGIP fraction (0.9 µg/mL final concentration) in 40 mM sodium acetate buffer pH 5.5 and 1% (w/v) azocasein in a final volume of 350 µL. After incubation at 37 °C for 30 min, proteolysis was stopped by the addition of 1 mL trichloroacetic acid (TCA) 10%. Precipitation was achieved by cooling at 0 °C for 20 min and it was centrifuged at 10,000x*g* for 5 min. An equal volume of NaOH (1 N) was added to the supernatant and the absorbance was recorded at 440 nm.

Glycosidase assay

Glycosidase activity was assayed by using sucrose (Sigma-Aldrich) as substrate. The reaction mixture for standard assay contained 10 µL of PGIP fraction (0.9 µg/mL final concentration) in 40 mM sodium acetate buffer pH 5.5 and 1% (w/v) sucrose in a final volume of 110 µL. After incubation at 37 °C for 30 min, glucose produced from the hydrolysis of sucrose was measured adding 1 ml of reagent A (Enzymatic glycemia AA, Wiener Lab., Argentina) containing glucose oxidase (10 kU/l), peroxidase (1 kU/l), 4-aminofenazone (0,5 mM), PBS (100

mM, pH 7) and 4-hydroxibenzoate (12 mM). After 5 min at room temperature the absorbance was recorded at 505 nm.

Toxicity and genotoxicity assay of inhibitory protein

General toxicity of protein using Artemia salina

The *A. salina* lethality test was employed as a general toxicity test, using lethal concentration (LC_{50}) as an indicator of the short-term poisoning potential. The LC_{50} value was defined as the amount of inhibitor that causes the death of 50% of nauplii. *A. salina* encysted eggs were incubated in 50 mL of filtered sea water in a small container divided into two compartments. The shrimp eggs were added to the covered compartment and a lamp was placed above the open side of the container in order to attract hatched shrimps through perforations in the partition wall. After 24 h incubation, the mature shrimps (nauplii) were collected with a Pasteur pipette. Ten nauplii were added to well of 96-well plate containing a dilution of the inhibitory protein (between 0 and 5 µg/mL). Control treatment containing only sea water was included in the experiment under the same conditions used in the others treatments. After 24 h incubation under light, the number of dead and surviving brine shrimps in each well was counted. The accuracy of the method was evaluated using at least three replicates of each concentration of protein. The positive control was potassium dichromate.

Phytotoxicity assay

Lactuca sativa L. var. Gran Rapid seeds were selected for seed germination and elongation assays. Seeds were placed on filter paper disks (Whatman No. 1) set at the bottom of 24 well-plates (10 per well) and treated with different inhibitor concentrations (up to 5 µg of protein). Growth tests were carried out using seedlings with primary roots equal to 2 cm. The seedlings were placed on Petri dishes (10 per well). Negative controls were performed with sterilized distilled water. Petri dishes from germination and growth treatments were maintained for 120 h and 48 h, respectively, at 25 ± 1°C. Treatments were replicated four times.

Genotoxicity assay using the Ames test

Genotoxicity assays were performed following the procedure described by **Maron and Ames (1983)**. *Salmonella thyphimurium* TA98 and TA100 were cultured overnight in Oxoid Nutrient Broth for 12 h. Different dilutions of inhibitory protein were added to 2 mL of top agar and 0.1 mL of each bacterial culture, and poured onto a plate containing minimum agar. The plates were incubated at 37 °C for 48 h and the histidine revertant colonies were counted. All experiments were performed in triplicate with at least two replicates. A two-fold or greater increase in the number of revertants exposed to the test material over spontaneous reversion rate was considered a positive mutagenic result. Negative and positive controls were included in each assay. The mutagen used as positive control was 4-nitro-*o*-phenylenediamine (10 µg per plate), which is a direct-acting mutagen.

RESULTS AND DISCUSSION

Isolation and characterization of PG

PGs, the most abundant and extensively studied of the pectinolytic enzymes, typically exist in multi-gene families and may have both *endo* (**Benen et al., 1999**) and *exo* activities (**Sakamoto et al., 2002**). Previously, endo-PG production by *G. candidum* was reported by **Barash et al. (1984)**. Nevertheless, recently **Nakamura et al. (2008)** reported that there are two *G. candidum* types: pathogenic and non-pathogenic isolated. In addition, they reported that PGs from *G. candidum* citrus type were responsible for its pathogenicity to citrus fruits (**Nakamura et al., 2003**). But, PGs from *G. candidum* non-citrus type had no pathogenicity (affinity to protopectin; protopectinase activity) (**Nakamura et al., 2003**). In this work, in order to distinguish between the citrus and non-citrus types of *G. candidum*, a first pathogenicity test (inoculating the fruit with the fungus) was carried out. Then, *G. candidum* isolated from infected fruit was cultured in a media with pectin in order to promote the PG production. The highest polygalacturonase activity was obtained after 10 days of incubation of the fungus. PG-543 was purified to homogeneity from the culture supernatant of *G. candidum* after three steps of purification that included ion-exchange and size exclusion chromatographic techniques (Table 1).

Table 1 Purification scheme of polygalacturonase from *G. candidum*.

	Volume (ml)	Activity EU/ml	Protein µg/ml	Specific activity (EU/mg)	Purification (fold)	Yield (%)
Crude extract	280	914.46	178.88	5.11	1.0	100.0
Amonium sulphate precipitation	12	1540.19	229.40	6.71	1.3	7.2
DEAE Sepharose CL6B	39	444.51	5.72	77.71	15.2	6.8
Sephadex G-150	75	231.30	2.36	98.01	19.2	6.8

The enzyme was recovered from the liquid culture medium by ammonium sulphate precipitation. Then, two sequential chromatographic steps in DEAE-Sepharose CL6B and Sephadex G-150 (Table 1) were performed and one peak of PG-543 activity was obtained after last step of purification (Fig. 1.A and 1.B).
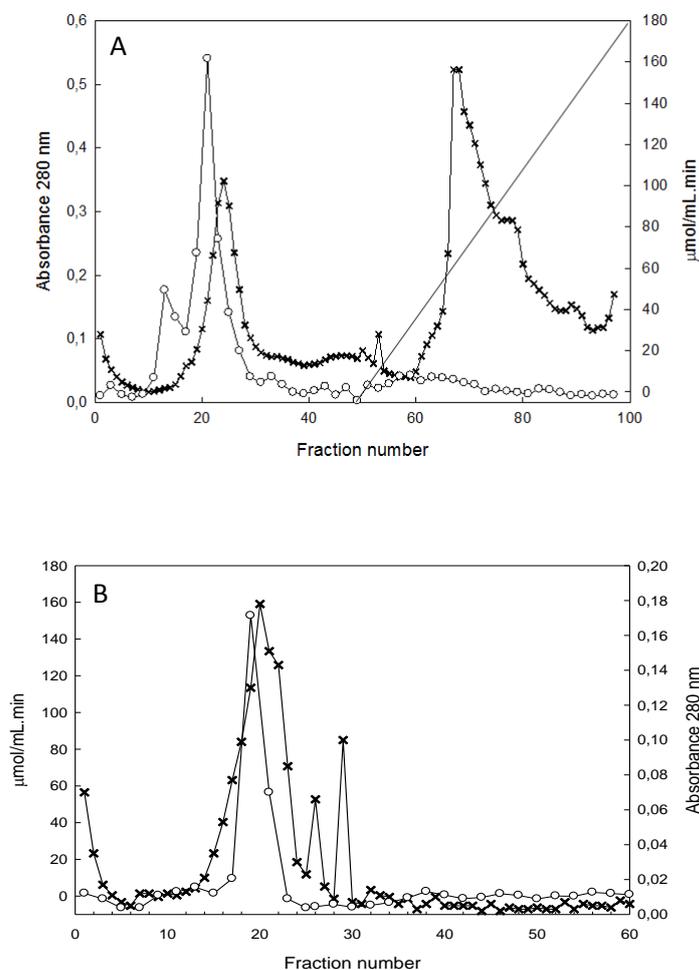
Figure 1. Elution profile of PG-543 from *G. candidum* in (A) DEAE-Sepharose CL 6B column (10 x 1.5 cm), eluted with a linear gradient of NaCl (0–1 M); (B) Sephadex G-150 column 20 cm×2 cm i.d.), eluted with 50mM sodium acetate buffer, pH 5.5. -o- PG activity (µmol/mL.min), -x- absorbance at 280 nm.

The enzyme was purified about 19-fold with an increase in specific activity up to 98 EU/mg, which represented a 6.8% yield (Table 1). SDS-PAGE analysis revealed the presence of a polypeptidic band of approximately 26 kDa (Fig. 2).
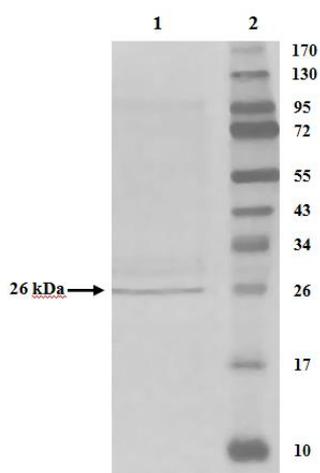
Figure 2 SDS-PAGE (15%) analysis of the purified PG-543 from *G. candidum*. Proteins were stained using the silver staining procedure. Lane 1: Fraction from Sephadex G-150 column showing purified PG-543 (1 μg of protein). Lane 2: Molecular mass standard. The molecular mass standards are reported in kDa.

Similar results were reported for the PGs produced by other strains of *G. candidum*. **Barash et al. (1984)** reported that the MW of the endo-PG from *G. candidum* was 38 kDa. **Nakamura et al. (2001)** also reported that the presumptive MW of PG S31PG1 from *G. candidum* citrus race S31 was 35.5 kDa on the basis of its amino acid sequence. Generally, PGs isolated from different microorganisms showed molecular weights around 30 kDa (**Gadre et al., 2003; Niture and Pant, 2004; Mohamed et al., 2006; Yuan et al., 2012**).

The purified PG showed maximum activity at pH 5.5 and at a temperature of 37 °C. The effect of pH on the stability of PG-543 from *G. candidum* was investigated by incubating the enzyme at different pH values for 45 min. The results showed that the enzyme was very stable at pH 5.5, but did not retain its activity at lower and higher pH values. These results are consistent with those reported for many microbial PGs (**Serrat et al., 2002; Aminzadeh et al., 2006; Mohamed et al., 2006; Dinu et al., 2007; Kant et al., 2013**). In general, most PGs are acidic and/or neutral enzymes (optimal pH between 3 and 6.5).

The purified PG-543 showed and optimum temperature of 37 °C and was stable at low and media temperatures (20 to 45 °C), but was severely affected at higher temperatures (Fig. 3). These results are similar to those informed for the PGs

from fungi *Tetracoccosporium* sp. and *Trichoderma harzianum*, with optimal temperatures of 40 °C (**Aminzadeh et al., 2006; Mohamed et al., 2006**). However, other authors reported optimum temperatures for PG activities between 50 and 55 °C (**Serrat et al., 2002; Cho et al., 2012; Maller et al., 2013; Yadav et al., 2013**).
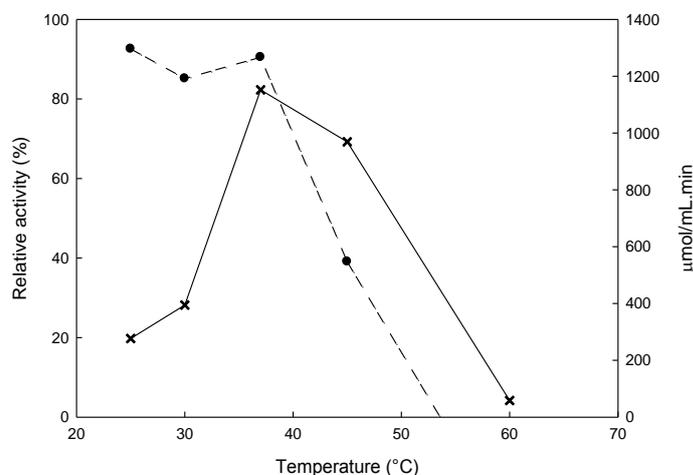
Figure 3 Temperature optimum (-x-) and temperature stability (-●-) of PG-543. The study of substrate specificity for *G. candidum* PG-543 was made by using citric pectin and sodium polygalacturonate as substrates. The results showed that PG-543 was specific only toward sodium polygalacturonate. Analysis of the hydrolysis products by TLC and enzyme activity assay using Ruthenium red method confirmed endo-polygalacturonase activity of PG-543. The kinetic constants of the purified PG were calculated by fitting the data to a linear regression on double-reciprocal plot. The kinetic was Michaelian with linear Lineweaver–Burk plot (data not shown). K_m value for sodium polygalacturonate was 2.94 mg/mL and the corresponding V_{max} was 227.3 μmol/mL min. The estimated K_{cat} was 41.7 s^{-1} (Table 2).

Table 2 Kinetic parameters of *G. candidum* PG-543[a] in the absence and presence of PGIP from lemon.

Sample	Kinetic Parameters				
	K_m (mg mL^{-1})	V_{max} (μmol mL^{-1} min^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (mg mL^{-1} s^{-1})	K_i (μg mL^{-1})
G. candidum PG-543	2.94	227.3	41.7	14.18	N.D.[c]
G. candidum PG-543 + PGIP	2.94	11.7[b]	N.D.	N.D.	0.125

[a] The PG activity was measured in a mixture containing PG-543 enzyme (1.15 EU) and sodium polygalacturonate (2-30 mg/mL) as substrate in 0.2 M sodium acetate (pH 5.5; 37°C), and containing or not different PGIP concentration (0.2-1.2 μg/mL).
[b] 1.2 μg/mL of PGIP
[c] N.D., not determined.

In previous studies, the K_m values for fungal endo-PGs were reported between 0.08 and 14.0 mg/mL using sodium polygalacturonate as substrate (**Gummadi and Panda, 2003; Mohamed et al., 2006; Yang et al., 2011; Kant et al., 2013**). The K_m value of PG-543 using sodium polygalacturonate was near to K_m values ranged from 2.5 to 4.1 mg/mL described for fungal endo-PGs from *Aspergillus niger* (**Mohamed et al., 2006; Arotupin et al., 2012**) and *Fusarium oxysporum* (**Al-Najada et al., 2012**).

Isolation of Polygalacturonase-inhibiting protein (PGIP) from lemon albedo and characterization of the PGIP-PG interaction

As a first approach to study the disease resistance mechanism in citrus, we focused on the study of polygalacturonase-inhibiting proteins (PGIPs). These proteins inhibit the activity of fungal PG, which are considered to be an important factor for pathogenesis (**De Lorenzo et al., 2001; Reignault et al., 2008**). PGIPs are cell wall proteins of vegetative as well as fruit tissues (**Favaron et al., 1994; Isla et al., 2002; Schacht et al., 2011; Ordoñez et al., 2012**). In the present work we showed the isolation of PGIP from lemon albedo. PGIP was partially purified in order to remove compounds that may interfere with the inhibition of the PG by

PGIP. The PGIP was extracted from ripe lemon albedo and was partially purified by DEAE-Sepharose chromatography. The protein fraction displaying PGIP activity was submitted to SDS–PAGE analysis. Protein bands with estimated molecular masses from 20 to 35 kDa and 70 kDa were observed (Fig. 4).
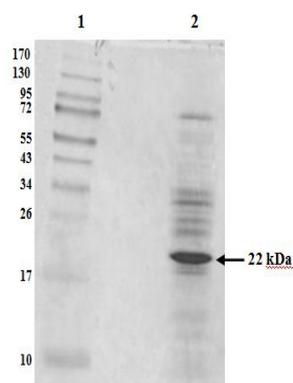
Figure 4 SDS-PAGE (15 %) analysis of partially purified PGIP extract from lemon albedo. Proteins were stained using the silver staining procedure. Lane 1: Molecular mass standard. The molecular mass standards are reported in kDa. Lane 2: Fraction from DEAE-Sepharose CL 6B column containing PGIP.

The main polypeptidic band showed a molecular mass around 22 kDa. Molecular weights of PGIPs isolated from different sources are from 30 to 70 kDa (**James and Dubery, 2001; Katoh *et al.*, 2007; Fan *et al.*, 2010**). A previous work described a PGIP of about 70 kDa isolated from citrus leaves that was capable of inhibiting polygalacturonases from *Aspergillus niger* (**Katoh *et al.*, 2007**). The PGIP from lemon albedo was able to inhibit completely the activity of the *G. candidum* PG-543. The contact time between PGIP (0.9 µg/ml) and PG-543 enzyme (1.15 EU) necessary to produce 50% inhibition was between 20 and 40 min (Fig. 6.A). Since PGIP was partially purified, the presence of enzyme activities that could hydrolyze PG-543 was studied in fraction displaying PGIP activity. Both protease and glycosidase activities were not detected under the same conditions able to produce 50% inhibition of PG (data not shown). The absence of hydrolytic activities in PGIP fraction discards that the loss of PG activity was due to enzymatic degradation of the PG. Previous studies reported that PGIPs from citrus leaves and pearl millet were able to inhibit 60% and 38%, respectively, the activity of *Aspergillus niger* PGs (**Katoh *et al.*, 2007; Prabhu *et al.*, 2012**). The PGIP changed V_{max} of the enzyme (1.2 µg/mL of PGIP caused a reduction of about 90 % in the V_{max} of PG-543) but not affected K_m values of PG-543, suggesting that this protein acts as a noncompetitive inhibitor of the enzyme (K_i=0.125 µg/mL) (Table 2). Such like type of inhibition was described for PGIPs from beans and raspberry (**Lafitte *et al.*, 1984; Johnston *et al.*, 1993**). Though, different mechanisms of inhibition were reported for PGIPs, such as an apple PGIP that showed mixed-type inhibition and the competitive inhibition of pear PGIP (**Yao *et al.*, 1995; Isla *et al.*, 2002**).

The stability of PGIP was determined by incubating the inhibitor at different pH during 10 min and then, the inhibitor was put in contact with the PG-543 enzyme (Fig. 5.B). In these conditions, PGIP was stable in the pH range between 2 and 6, while at pH values greater than 6 the inhibitory capacity decreased. The PGIP was stable in the temperature range between 20 and 60 °C, demonstrating the thermal stability of this protein (Fig. 5.C).

A

B

C
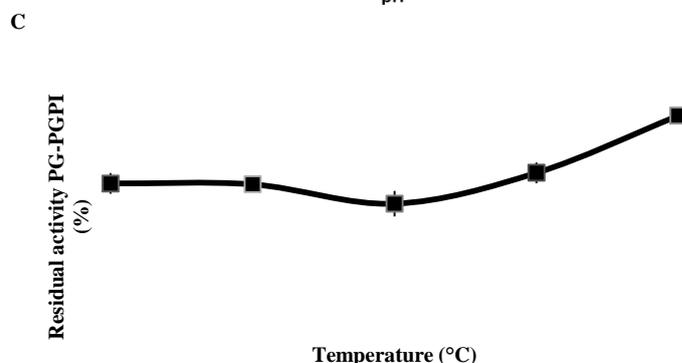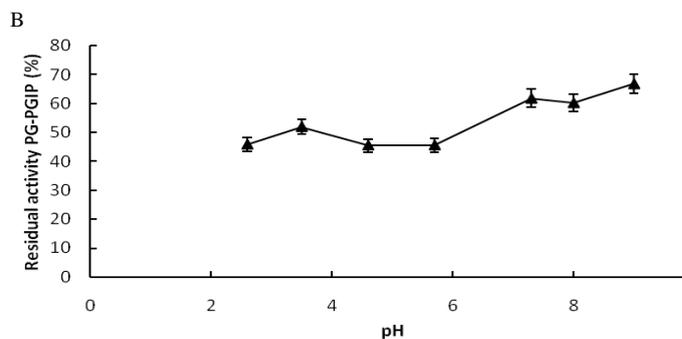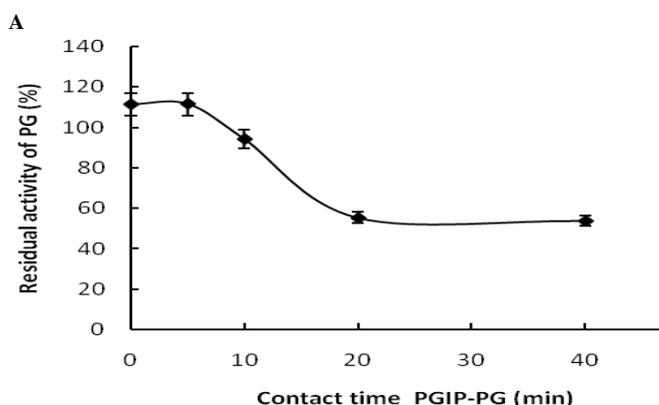
Figure 5 (A) Effect of interaction time between PGIP and PG-543 on the enzyme activity inhibition. (B) pH **s**tability of PGIP. (C) Thermal stability of PGIP.

Toxicity, phytotoxicity and genotoxicity studies

PGIP did not show toxic effect on *Artemia salina* at the concentration range in which it was active on PG enzyme. The PGIP was also assayed on germination of *Lactuca sativa* seeds and elongation of primary root. The germination was not affected by PGIP (up to 5 µg/ml). The growth of primary root in presence of PGIP was higher than the growth in its absence. Furthermore, mutagenicity was evaluated by the Ames assay. Table 3 shows the number of revertants/plate after the treatments with the isolated protein in two different strains of *S. typhimurium*. None of the doses (up to 5 µg/mL) were mutagenic in strains TA98 or TA100 under the conditions used in this assay. This result indicates the inexistence of mutagens that cause base pair substitution (detected in TA100) and frameshift (detected in TA98) mutations. The absence of mutagenicity for the protein preparations studied in the tested *Salmonella* strains indicates that DNA does not seem to be a relevant target for PGIP.

Table 3 Revertant/plate in the strains TA98 and TA100 of *Salmonella typhimurium* after treatment with various doses of PGPI.

Sample	µg /Plate	TA 98	TA 100
		Number of revertant/plate[a]	
PGIP°	1.25	25 ± 2	150 ± 10
	2.50	26 ± 3	158 ± 13
	5.00	33 ± 2	168 ± 32
Positive control[b]		1078 ± 89	963 ± 62
Negative control[c]		23 ± 1	133 ± 12

[a]Mean number of revertants [Mean of four plates ± S.D.]
[b]Mean number of revertants induced by reference mutagens 4-nitro-o-phenylenediamine (10 µg/plate) positive control
[c]The number of spontaneus revertants was determined in a assays without sample.

CONCLUSIONS

Plant cell wall-degrading enzymes are present in both pathogenic and nonpathogenic microorganisms and the role of each enzyme in infection depends on the combination of pathogen and host. In certain situations, some enzymes could be important for infection and others not. However, scientific evidence demonstrated that one type of cell wall-degrading enzyme, the PGs, play an essential role in the pathogenicity of pathogens that infect pectin-rich plants (**Isshiki *et al.*, 2000; Nakamura *et al.*, 2003**). *G. candidum* IEV 543 was able to produce a PG that catalyzes a hydrolytic cleavage of galacturonic acid polymers with low C-6 methylation grade (pectate). PG-543 could be a very important virulence factor in *G. candidum* that enables infection of fruit tissue. We have

shown that the proteinaceous inhibitor isolated from "lemon albedo" inhibited this enzyme. PGIP is suggested to be involved in the defense mechanism of lemon to *G. candidum*, performing as a hydrolase inhibitory protein. This PGIP could be used as a natural product to decrease the propagation of this pathogenic fungus, preventing the development of postharvest diseases.

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