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A colorimetric method to quantify endo-polygalacturonase activity

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ABSTRACT

We report a new colorimetric assay to quantify endo-polygalacturonase activity, which hydrolyzes polygalacturonic acid to produce smaller chains of galacturonate. Some of the reported polygalacturonase assays measure the activity by detecting the appearance of reducing ends such as the Somogyi–Nelson method. As a result of being general towards reducing groups, the Somogyi–Nelson method is not appropriate when studying polygalacturonase and polygalacturonase inhibitors in plant crude extracts, which often have a strong reducing power. Ruthenium Red is an inorganic dye that binds polygalacturonic acid and causes its precipitation. In the presence of polygalacturonase, polygalacturonic acid is hydrolyzed bringing about a corresponding gain in soluble Ruthenium Red. The described assay utilizes Ruthenium Red as the detection reagent which has been used previously in plate-based assays but not in liquid medium reactions. The new method measures the disappearance of the substrate polygalacturonic acid and is compared to the Somogyi–Nelson assay. The experimental results using lemon peel, a fern fronds and castor leaf crude extracts demonstrate that the new method provides a way to the quickly screening of polygalacturonase activity and polygalacturonase inhibitors in plant crude extracts containing high amounts of reducing power. On the other hand, the Ruthenium Red assay is not able to determine the activity of an exo-polygalacturonase as initial velocity and thus would allow the differentiation between endo- and exo-polygalacturonase activities.

1. Introduction

Polygalacturonases (endo-polygalacturonase, E.C. 3.2.1.15 and exo-polygalacturonase, E.C. 3.2.1.67) are enzymes that catalyze the hydrolytic cleavage of the polygalacturonic acid chain by introducing water across the oxygen bridge [1]. Endo-polygalacturonases hydrolyze polygalacturonic acid chains by a random mechanism of attack, whereas exo-polygalacturonases hydrolyze such chains by a terminal mechanism of attack. Polygalacturonases (PGs) are widely distributed among bacteria, yeasts, filamentous fungi and higher plants and could also be found in nematodes and insects [1,2]. Although the physiological functions of PGs are still under investigation, the involvement of PGs in the early stages of infection of plants by fungi has been demonstrated [2–4]. These enzymes have also attracted great interest in industry due to their growing number of biotechnological applications, from the clarification of fruit juices and wines to the preparation of oligosaccharides from pectic polymers [5–7].

Polygalacturonase inhibiting proteins (PGIPs) have been identified in the tissue of most plants. These cell wall-associated glycoproteins can effectively inhibit the fungal endo-PGs [8] and therefore they represent important elements in the defense mechanisms of the plant against fungal pathogens [4]. The accumulation of oligogalacturonides in plant tissues due to the inhibition of microbial PGs has also been proposed as a defense mechanism [9]. Thus, interest in PG inhibitors has increased recently because of their potential use as biotechnological tools for controlling phytopathogenic fungi [10–13].

The methods currently used to determine PG activity are based on the measurement of either the rate of increase of reducing groups or the decrease of reaction mixture viscosity as a result of the hydrolysis of the substrate during the course of reaction. Viscosity reduction can be determined using a viscosimeter connected to a detector system [14]. This assay compares reaction mixture viscosity after enzymatic hydrolysis with that of the same solution without enzyme; however, the viscosity reduction method was used with limited success and with the addition of the constraints arising from the need for special equipment [1]. On the other hand, the number of reducing groups generated during PG action on the polygalacturonic acid (PGA) can be measured by colorimetric methods like dinitrosalicylic acid reagent method [15], the arsenomolybdate-copper reagent method [16,17] or cyanoac-

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etamide method [18]. Nevertheless, these methods cannot be used to assess PG activity of extracts with high endogenous reducing power. Moreover, methods based on reducing group measurement are not useful for differentiating exo-PG from endo-PG activities.

Ruthenium Red (RR) is a cationic dye with six positive charges, which forms electrostatic bonds to the acidic groups of sugars. In this regard, RR is known to precipitate a large variety of polyanions by ionic interaction and its classical reaction with pectin is typical rather than specific [19]. RR dye interaction with polyuronic acids has been utilized to stain polyanionic structures. It was shown that the disruption of high negative charge density at the staining site by esterification reduces the intensity of the pectin staining by RR [20,21]; this pattern of staining has been used in the differential detection of PGA depolymerizing enzymes after isoelectric focusing and in the determination of pectin methyl esterase and PG activities in gel diffusion assays [22–26].

The objective of the present study was to develop an alternative colorimetric assay to quantify PG activity based on the interaction of RR dye with PGA, even in the presence of compounds with high reducing power. Furthermore, the use of the RR dye–PGA interaction is also attempted to achieve a method for the differentiation of endo- and exo-PG activities.

2. Materials and methods

2.1. Microorganism, growth conditions and enzyme preparations

The endo-PG from the fungus *Geotrichum candidum* F 4562 was used to standardize the enzyme assay. For enzyme production, *G. candidum* was grown on a pectin containing medium (peptone, 10 g/L and pectin, 30 g/L), without shaking, at 30 °C for 7 days. Cell-free supernatants were obtained by centrifuging the cultures at 21,000 × *g* for 15 min (4 °C). Supernatants (proteins: 3.15 μg/mL) were concentrated by precipitation with $(NH_4)_2SO_4$ (100% stn), dialyzed against 20 mM sodium acetate buffer pH 5.5 and then used as a source of endo-PG activity.

Exo-PG was obtained from carrot roots (*Daucus carota*) [27]. Carrots were purchased in a local supermarket and were ground and extracted with 20 mM sodium phosphate buffer pH 7.5, containing 1 M NaCl. This mixture was maintained for 3 h at 4 °C, filtered through two layers of gauze and centrifuged at 21,000 × *g* for 15 min at 4 °C. The supernatant was concentrated by precipitation with $(NH_4)_2SO_4$ (100% stn) and the pellet was extensively dialyzed against 20 mM sodium phosphate buffer pH 7.5. Enzyme solutions (proteins: 30 μg/mL) were centrifuged at 10,000 × *g* and filtered through a 0.45-μm micro-filter (nylon) before being used as an exo-PG source.

2.2. Polygalacturonase inhibitor preparation

Extracts with inhibitory activity against endo-PG of *G. candidum* were obtained from lemon peel (*Citrus limon*), fern frond (*Pteris deflexa*) and castor bean leaf (*Ricinus communis*).

Lemon peels were ground and then extracted with 20 mM sodium acetate buffer pH 5.5, containing 1 M NaCl, 1 mM 2-mercaptoethanol and 1% (w/v) polyvinyl-polypirrolidone (1 mL of buffer per g of plant material). This mixture was continuously stirred overnight at 4 °C, filtered through two layers of gauze and centrifuged at 21,000 × *g* for 15 min at 4 °C. The supernatant was concentrated by precipitation with $(NH_4)_2SO_4$ (100% stn) and the pellet was extensively dialyzed against 10 mM sodium acetate buffer pH 5.5 containing 1 mM 2-mercaptoethanol.

Castor bean leaves were ground and extracted with 50 mM sodium phosphate buffer pH 7.5 containing 0.5 M NaCl and 1 mM 2-mercaptoethanol (1 mL of buffer per g of plant material). The extract was filtered through two layers of gauze and centrifuged at 21,000 × *g* for 15 min at 4 °C. The supernatant was concentrated by precipitation with $(NH_4)_2SO_4$ (100% stn) and the pellet was extensively dialyzed against 10 mM sodium acetate buffer pH 5.5 containing 1 mM 2-mercaptoethanol.

Dried rhizomes from *P. deflexa* were ground in a grinder. Five grams of this material was macerated at 30 °C with shaking (50 rpm) with 25 mL 60% ethanol during 7 days. The hydroalcoholic extract was then filtered through two layers of gauze and centrifuged at 21,000 × *g* for 15 min at 4 °C. The supernatant was concentrated to dryness at reduced pressure and 40 °C. The sediment was resuspended in distilled water and the suspension was centrifuged at 21,000 × *g* for 15 min at 4 °C. The resulting supernatant was considered aqueous solution of secondary metabolites from *P. deflexa* rhizomes and kept at −20 °C until used.

2.3. Dye solution preparation and properties

Stock solution of Ruthenium Red (Sigma R-2751) was prepared by dissolving 5 mg in 1 mL of distilled H_2O and kept at 0 °C until use. Colorimetric experiments were carried out in a Beckman UV–visible recording spectrophotometer (Beckman DU 650 spectrophotometer, USA) at 535 nm with a 1-mL path-light cuvette.

The absorbance of increasing RR concentrations from 8.3 to 67 μg/mL was investigated in 1.33 mM sodium acetate (pH range 3.5–5.5), sodium phosphate (pH range 6.5–7.5) and NaOH–glycine (pH range 8.5–9.5) buffers. Also, the interaction of increasing PGA concentrations from 0 to 0.01% (w/v) with various RR concentrations (from 8.3 to 67 μg/mL) was studied in 1.33 mM sodium acetate (pH 5.5).

2.4. Enzyme assays

Unless otherwise noted, the reaction mixture for standard assays for the screening of PG activity contained 5–15 μL of the *G. candidum* enzyme preparation (endo-PG) or 40 μL of the carrot roots enzyme preparation (exo-PG) in 80 mM sodium acetate buffer pH 5.5 and 0.187% (w/v) PGA in a final volume of 100 μL. Enzyme reactions were started by the addition of the substrate to the reaction mixtures and incubated at 40 °C during different times. The time course of the endo-PG reaction was evaluated incubating the reaction mixtures up to 20 min, whereas exo-PG preparation was assayed up to 5 h. The effect of PGA concentration on endo-PG activity was determined in reactions with increasing amounts of PGA in the range 0–0.187% (w/v) and 10 μL of crude enzyme preparation incubated for 10 min.

PG activities were measured either as the amount of the reducing ends generated during the enzyme reaction determined by the Somogyi–Nelson method or as the remaining substrate after the enzyme hydrolysis by the novel RR dye method. The endo- and exo-PG activities were confirmed by the detection of galacturonic acid by thin-layer chromatography (TLC).

2.4.1. Somogyi–Nelson method

The amount of PGA hydrolyzed was determined by measuring the increase in reducing groups during the reaction course using D-galacturonic acid as a standard; after incubation, the enzymatic reaction was stopped by the Cu alkaline reagent [17] and reducing power was measured according to Nelson [16]. One enzyme unit was defined as the amount of enzyme required to release 1 μmol of reducing groups per minute under the standard assay conditions.

2.4.2. Ruthenium Red dye method

The amount of hydrolyzed substrate was calculated measuring the remaining PGA after PG action. After the incubation of the enzymatic reaction, the mixture was carried to a volume of 3 mL with distilled water, then 40 μL of RR stock solution was added and the mixture was homogenized. The mixture was carried to a final volume of 6 mL with distilled water, homogenized and centrifuged at 1000 × *g* for 5 min at room temperature in order to separate the precipitated RR–PGA complex. Control mixtures without enzyme and without substrate were also incubated. One unit of enzyme was defined as the amount of enzyme required to hydrolyze 1 μg of PGA to smaller fragments unable to precipitate with the dye per minute under the standard assay conditions.

2.4.3. Analysis of hydrolytic pectic enzyme reaction products

Reaction products resulting from polygalacturonic acid due to the activity of the PG preparations from *G. candidum* or *D. carota* were analyzed by TLC. Reaction mixtures containing 200 μL of the *G. candidum* or *D. carota* enzyme preparation in 80 mM sodium acetate buffer pH 5.5 and 0.2% (w/v) PGA in a final volume of 1000 μL were incubated up to 5 h at 40 °C. Enzyme reactions were started by the addition of the substrate and stopped by heating at 100 °C during 3 min. Reaction mixtures were desalted with Amberlite MB-3. Aliquots of 20 μL from different incubation times were applied on thin-layer silica sheets (Merck) and developed for 12 h with n-butanol–pyridine–water (6:4:3, v/v/v) as solvent system. The chromatograms were air dried and sprayed with diphenyl amine/aniline/phosphoric acid solution [28]. Products containing uronic acid residues appeared as blue spots.

2.5. Effect of plant glycosidase inhibitors on G. candidum PG

The inhibitory effects of lemon peel and castor bean leaf extracts on PG activity were studied adding from 0 to 807 μg of protein of each of them to the reaction mixture containing 15 μL of *G. candidum* enzyme preparation and incubating for 20 min at room temperature. Enzyme reactions were started with the addition of the substrate and incubated as stated before. The inhibitory activity of the fern extract was assayed in the same conditions using 215 μg of phenolic compounds.

2.6. Protein determination

Protein concentration was determined according to Ref. [29] using bovine serum albumin as a standard.

2.7. Phenolic compounds determination

Phenolic compound's concentration was determined according to Ref. [30] using galic acid as a standard.

2.8. Reagents

All reagents were of analytical or microbiological grade and purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstad, Germany).

2.9. Statistical analysis

Data are represented as mean ± standard deviation. The statistical tests were carried out by analysis of variance (one-way ANOVA) and the post-test of Tukey, using a probability level of less than 5% ($p < 0.05$).

3. Results

Ruthenium Red (RR) showed maximum absorption at 535 nm either in distilled water or sodium acetate, NaOH–glycine or sodium phosphate buffer solutions. The absorption increase with increasing RR concentrations (from 8.3 to 67 μg/mL) was linear in the assayed media ($r^2 = 0.99$).

The addition of RR to PGA solutions resulted in the formation and precipitation of a dye–polysaccharide complex. RR solutions of concentrations between 8.3 and 67 μg/mL were assayed with different solutions of PGA at pH 5.5. When the RR concentration was equal to PGA concentration, almost all the dye formed a complex with PGA and precipitated. However, when the RR concentration was higher than the concentration of PGA, free dye remained in the supernatant. In this case, the absorbance values due to the free RR in the supernatant correlated negatively with PGA concentrations ($r^2 = 0.99$), being higher the absorbance at lower concentrations of PGA (Fig. 1, inbox). The replot of PGA–RR calibration curve showed a linear response with positive slope (Fig. 1). The replot was obtained as the subtraction between the A_{535} of the solution without PGA and the A_{535} of a solution with PGA (ΔA_{535} vs. μg of PGA). This replot can be used to quantify PGA concentration. Hence, it could be useful to determine PG activity expressed as μg of PGA hydrolyzed per minute. An increase in the absorbance implies a higher amount of hydrolyzed PGA.

The interaction of RR with the polysaccharide was affected by pH values, and the 1:1 relation between dye and polysaccharide concentrations changes at a pH values higher than 5.5. Thus, in order to carry out the PGA determination the RR concentration was adjusted to 33 μg/mL for pH values between 3.5 and 5.5, and to 50 μg/mL for pH values between 6.5 and 9.5 (Table 1). The RR solution (33 μg/mL) allowed the quantification of PGA in the range of 0–31.2 μg/mL at pH 5.5. PGA concentrations higher than 31.2 μg/mL required higher amounts of dye, which was inconvenient for the assay due to the great increase in the supernatant absorbance.

Table 1
Concentration of Ruthenium Red adjusted in order to quantify PGA in the range of 0–31.2 μg/mL according the solution pH value.

pH	Buffer[a]	Ruthenium Red (μg/mL)[b]	Linear regression equation[c]
3.5	Acetate	33	$y = 0.0047x - 0.0582$
5.5	Acetate	33	$y = 0.0058x - 0.0469$
6.5	Phosphate	50	$y = 0.0063x - 0.0294$
7.5	Phosphate	50	$y = 0.0067x - 0.0422$
9.5	NaOH–glycine	50	$y = 0.0074x - 0.0644$

[a] The concentration of each buffer in the reaction mixture (100 μL) was 80 mM; the concentration of each buffer in the final volume of the assay (6 mL) was 1.3 mM.
[b] Concentration of Ruthenium Red which precipitates with 31.2 μg/mL PGA in a final volume of 6 mL (equivalent to 187 μg in 100 μL mixture reaction volume).
[c] Equation obtained from the linear regression analyses made to each calibration curve at each pH studied. The correlation coefficient (r^2) of each equation was ≥0.99.

With the purpose of determining the usefulness of the assay in measuring endo- and exo-PG activities, enzymes from a fungus culture and from carrot root tissues were isolated. The pattern of action of PG activities (endo- or exo-cleaving enzyme) was evaluated by the analysis of the reaction products by TLC. The PG from carrot cleaved the polymer in an exo-fashion and generated

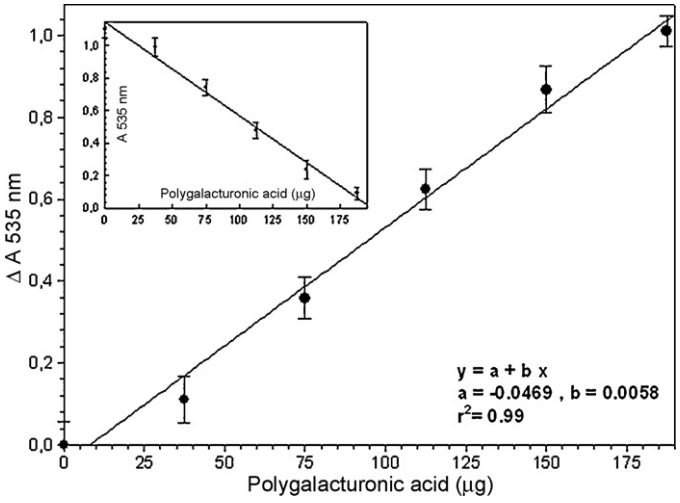

Fig. 1. Replot of the calibration curve of polygalacturonic acid with Ruthenium Red. Data are averages ± SD ($n = 4$). ΔA_{535} nm is the absorbance of the precipitated RR–PGA complex that was calculated according to the following equation: $\Delta A = A_0 - A_x$; where A_0 represents the absorbance of the solution without polygalacturonic acid and A_x, the absorbance of the solution with a determined amount of PGA. Inbox: calibration curve of polygalacturonic acid with Ruthenium Red. The absorbance of the Ruthenium Red in the supernatant was measured at 535 nm.

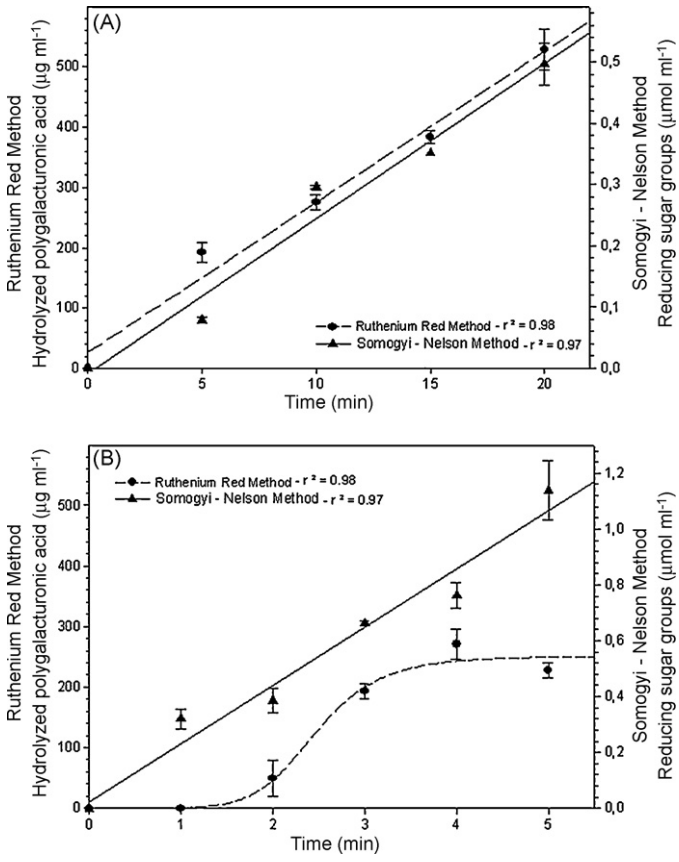

Fig. 2. (A) Time dependence of hydrolysis of polygalacturonic acid by *G. candidum* endo-polygalacturonase determined using Ruthenium Red dye method (●) and Somogyi–Nelson method (▲). Enzyme reaction was carried out at 40 °C in 80 mM sodium acetate buffer pH 5.5 for up to 20 min using 5 μL of endo-polygalacturonase crude extract. Data are averages ± SD ($n = 3$). (B) Time dependence of hydrolysis of polygalacturonic acid by carrot exo-polygalacturonase determined using Ruthenium Red dye method (●) and Somogyi–Nelson method (▲). Enzyme reaction was carried out at 40 °C in 80 mM sodium acetate buffer pH 5.5 for up to 5 h using 40 μL of exo-polygalacturonase crude extract. Data are averages ± SD ($n = 3$).

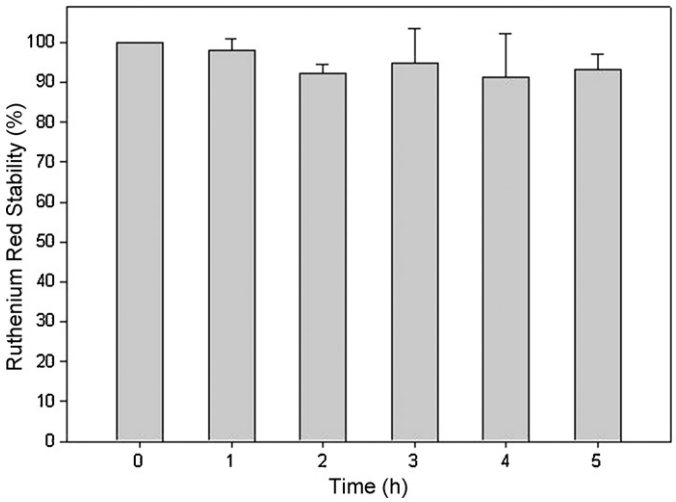

Fig. 3. Ruthenium Red stability over the time. Endo-polygalacturonase activity was measured using the Ruthenium Red dye method. After performing the assay the absorbance of the colored supernatant was evaluated during 5 h.

only galacturonic acid, as determined from Rf values previously reported [27] and from comparisons with the monomer standard (not shown). *G. candidum* enzyme generated a series of oligomers indicating that the polymer was cleaved in a random and endo-fashion. Enzyme preparations incubated with RR in the absence of substrate were unable to produce precipitate even under different incubation times. These results indicated that PG preparations lacked of polyanionic compounds capable to precipitate with RR and demonstrated the specificity of the interaction between RR and polyuronic acid in our assay conditions.

The effect of incubation time on enzyme activities was evaluated at pH 5.5 using both the Somogyi–Nelson and RR dye methods (Fig. 2A and B). Both techniques showed that the reaction product concentrations increased linearly until 20 min for endo-PG from *G. candidum*. Carrot exo-PG activity evaluated by Somogyi–Nelson method showed a linear response with incubation time. However, the RR method was not useful for determining the exo-PG activity since a sigmoidal response was observed (Fig. 2B). The color developed in RR assays was stable during 5 h at room temperature (Fig. 3) indicating that the RR dye precipitated all residual PGA.

The determination of the effect of substrate concentration on endo-PG activity demonstrated similar saturation kinetics when it was measured by Somogyi–Nelson or by RR method (Fig. 4A and B). Indeed, results from both methods showed that a PGA concentration of 0.15% was sufficient to achieve substrate-saturated enzyme reactions.

The RR assay was also used to determine the inhibitory activity of lemon peel, castor bean leaf and fern frond extracts on endo-PG activity from *G. candidum*. The fern frond extract was able to produce endo-PG inhibition (maximal inhibition of 20% at 200 μg of phenolic compounds). The PG inhibitory activity of lemon peel extract was higher than that of castor bean leaf extract at low protein concentration (Fig. 5). Our results show that the method could be useful to measure the degrees of enzyme inhibition due to small increments of protein concentrations and that the PG-inhibitor preparations do not interfere with the RR assay.

4. Discussion

The quantitative determination of PG activity is routinely carried out by measuring the amount of reducing ends released from the substrate as a result of its hydrolysis [1] and the reaction rate

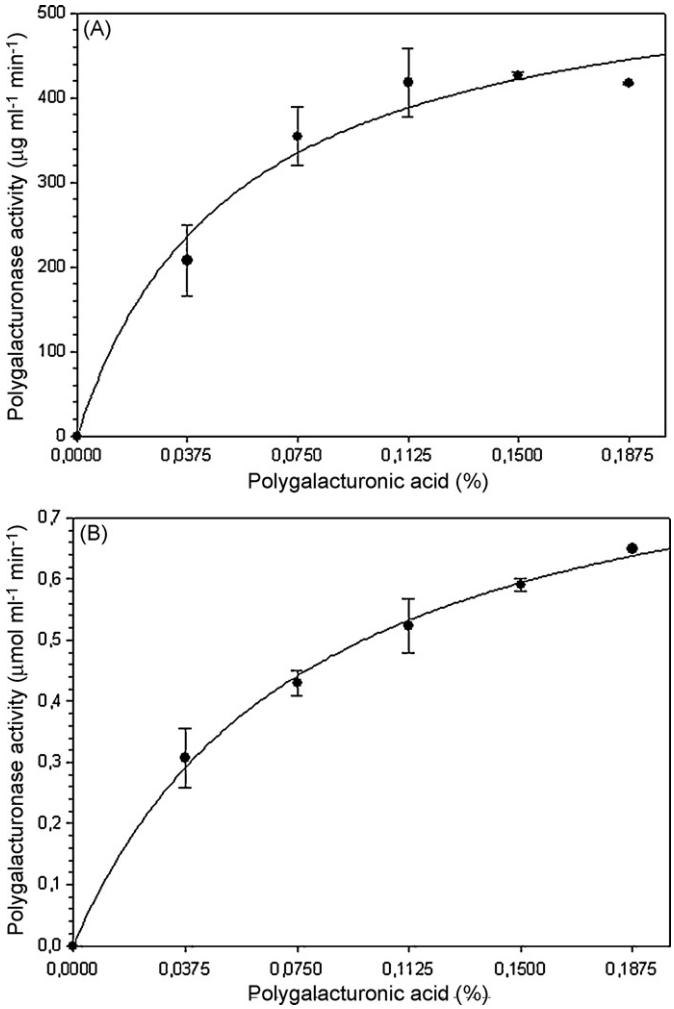

Fig. 4. Effect of polygalacturonic acid concentration on *G. candidum* endo-polygalacturonase activity measured using Ruthenium Red dye method (A) and Somogyi–Nelson method (B). Endo-polygalacturonase (10 μL of *G. candidum* crude extract) was incubated at 40 °C in 80 mM sodium acetate buffer pH 5.5 for 10 min with increasing amounts of polygalacturonic acid in the range 0–0.187% (w/v). Data are averages ± SD ($n = 4$).

being expressed as μmol of reducing groups released per minute. However, these methods have some drawbacks such as their use for the screening of proteins or secondary metabolites with PG inhibitory activity from crude or concentrated plant extracts, which usually have high levels of reducing power. On the other hand, plate assays that involve the use of RR dye or iodine in order to determine pectinolytic activity and its inhibitory activity are not affected by a reducing environment. However, in such cases the reaction rates are expressed as diameter of staining or destaining zones [25,26] and their sensitivity depend on the gel thickness and on the diffusion ability of the enzyme and/or inhibitor preparations within the plate.

Although the new RR dye method is adequate to determine PGA depolymerizing activities, like PGs or PGA lyases, the use of acidic pH in the assays ensures the selective determination of PG activity [23]. The utilized enzymes in order to assay the RR method were obtained from the phytopathogenic fungus, *G. candidum* (endo-PG) and from a plant source, *D. carota* (exo-PG). The depolymerase type was determined by TLC assays of the reaction products. Besides, exo-PG activity from carrot root tissues was reported to be the unique PGA depolymerase present in these tissues [27].

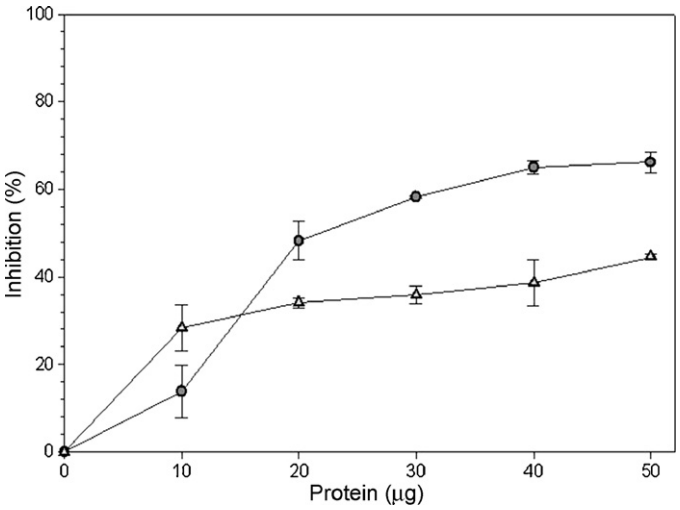

Fig. 5. Inhibition of *G. candidum* endo-polygalacturonase activity by increasing amounts of lemon peel (○) and castor bean leaf (△) extracts determined using the Ruthenium Red assay. The inhibition was studied incubating the enzyme (15 μL of *G. candidum* crude extract) with different amounts of each extract in 80 mM sodium acetate buffer pH 5.5 for 20 min at room temperature. Then, enzyme reaction was started adding 0.187% (w/v) polygalacturonic acid and the reaction mixture was incubated at 40 °C for 10 min. The inhibition activities of the extracts were expressed as percentage of inhibition of the endo-polygalacturonase activity (436 μg/mL min). Data are averages ± SD ($n = 3$).

Our results showed that the interaction between RR and PGA was not influenced by the other components of the reaction mixture. Moreover, the complex was stable at least for 5 h. The binding of the RR dye to PGA was not affected by reducing power of plant extracts. Hence, the new RR method could be useful to assay PG activity from plant with high amounts of reducing sugars or other compounds with reducing power.

The technique was used to determine the time course of the reaction and the effect of substrate concentration on the reaction rate of *G. candidum* endo-PG activity. The results from the colorimetric assay described here were similar to those obtained with the Somogyi–Nelson method. The inhibition ratio of endo-PG activity caused by lemon peel extracts was also similar with both methods. Furthermore, the reliability of the new performed spectrophotometric assay for the endo-PG measurement was also supported by the sensibility of the method to small variations of protein concentration of the enzyme inhibitors.

Moreover, the RR method allowed the rapid differentiation between endo- and exo-PGs, which cannot be carried out by measuring reducing ends. Due to the activity of exo-PG on PGA, the substrate size decreases and the same occurs by the endo-PG action. In both situations the remaining polymer was able to precipitate with the RR dye. Nevertheless, the remaining PGA by exo-PG action retained this ability during a longer time than in reactions catalyzed by endo-PG. Consequently, the activity of exo-PG could not be determined by RR method up to 1 h of incubation, while endo-PG activity was evaluated in 10 min. The Somogyi–Nelson method was useful both to determine the exo- and endo-hydrolase activities.

The linear response of endo-PG activity could be consequence of range of molecular weight of substrate (25,000–50,000) while the sigmoid response for exo-PG activity suggested a cooperative effect (many substrate molecules simultaneously could reach a size unable to precipitate with the RR dye).

In conclusion, this is an alternative, inexpensive and reliable method based on RR dye interaction with PGA for the quantitative estimation of an endo-PG activity, even in the presence of high level of reducing power. The assay is easy to handle and requires reduced execution time in comparison with other methods.

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