BIOLOGIA PLANTARUM 42 (4): 609-614, 1999

Purification and characterization of an endopolygalacturonase produced by *Sclerotinia sclerotiorum*

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Abstract

An endopolygalacturonase (endo-PG), was purified from the culture medium of a local isolate of *Sclerotinia sclerotiorum* with ammonium sulphate precipitation, cation exchange chromatography and gel filtration. The purified endo-PG had a molecular mass of approximately 18 kDa estimated by gel filtration. The isoelectric point was determined by isoelectric focusing to be approximately 8, suggesting that PG II possesses a net positive charge at physiological pHs. The pH optimum for the enzyme was at pH 4.5. The endo-PG showed essentially the same affinity for pectin and polygalacturonic acid as substrates.

Additional key words: enzyme production, galacturonic acid, oligogalacturonides, pectin-hydrolyzing enzyme.

Introduction

The penetration and colonization of plant tissues by microorganisms often involves the degradation of cell wall polymers. Plant pathogenic fungi and bacteria produce a wide array of cell wall-degrading enzymes. The importance of one of these enzymes, endopolygalacturonase (endo-PG; EC 3.2.1.15) in pathogenesis is well established. (Collmer and Keen 1986, Rodriguez-Palenzuela *et al.* 1991). Endo-PGs disrupt the structure of plant cell walls, allowing fungal colonization of plant tissue (English *et al.* 1972). However, it may also activate plant defence responses by releasing plant cell wall fragments (Bishop *et al.* 1981, Robertsen 1987, De Lorenzo *et al.* 1994).

Sclerotinia sclerotiorum causes head and basal rot, diseases which destroyed sunflower. In order to determine the role of endo-PG during pathogenesis, we report the production, purification and characterization of an endo-PG from *Sclerotinia sclerotiorum*.

Received 29 December 1998, *accepted* 3 June 1999.
Acknowledgements: This work was supported by grants of International Foundation for Sciences, Research Grant Agreement C/2411 1 and C.O.N.I.C.E.T, PIP No. 4340
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Materials and methods

Polygalacturonase (PG) production and purification: *Sclerotinia sclerotiorum* (local isolate) was grown in Maxwell liquid medium (Maxwell and Lumsden 1970). Sodium polygalacturonate and glucose were used as carbon source (0.5 and 2 % m/v, respectively). At daily intervals, samples of culture were withdrawn. The fluids separated from the mycelium by filtration through *Whatman No. 1*, were used for determination of enzyme activity.

PG was only purified from culture filtrates obtained 7 d after inoculation. The filtrate was concentrated about ten fold by 80 % ammonium sulphate precipitation. The concentrated enzyme solution was then dialysed overnight against 20 mM sodium acetate buffer (pH 5.0), and applied to a column of *MS HR 5/5* (*Pharmacia*, Uppsala, Sweden) for cation exchange chromatography. The column was eluted with a linear gradient of NaCl (0 - 0.5 M) in the same buffer, at a flow rate of 1 cm^3 min^{-1}. MS-cation exchange chromatography was the critical step which resulted in splitting of the PG activity into two components, which eluted at 130 and 280 mM of a linear NaCl gradient. The two isoenzymes detected were called PG-I and PG-II. This step resulted in significant purification of the PG enzymes. Taking account that only endo-PGs activate plant defence responses and that preliminary experiments (not shown) had shown that PG-I was an exo-PG, we report here only the characterization of PG-II. Minor contaminating proteins which remained after MS-cation exchange chromatography were separated from PG-II by gel filtration on *Superose 12 HR 10/30* (*Pharmacia*), equilibrated with 50 mM sodium acetate, pH 5.0. Fractions (0.001 cm^3) were collected during elution at a flow rate of 1 cm^3 min^{-1}.

Enzyme assay: PG activity was determined as the increase of reducing end-groups with time using 0.5 % (m/v) polygalacturonic acid (PGA; *Sigma*, St. Louis, USA) as substrate, in sodium acetate buffer (100 mM, pH 4.6). Reducing end-groups were measured by the method described by Milner *et al.* (Milner *et al*, 1967) using D-galacturonic acid (*Sigma*, St. Louis, USA) as a standard.

Fractionation of oligogalacturonides from PGA: PGA (0.5 %) was incubated with endo-PG in 50 mM acetate buffer, pH 4.6, at 30 °C. After 0, 2 and 5 h, aliquots of the incubation mixture were removed and analyzed by Fast Protein Liquid Chromatography (FPLC) using an anion-exchange *Mono Q HR 5/5* column (*Pharmacia*) equilibrated with 50 mM Tris-HCl buffer, pH 8. The column was eluted with a linear gradient of NaCl (0 - 0.5 M) in the same buffer. The fractions (0.5 cm^3) were assayed by the method described by Milner *et al.* (1967).

Results and discussion

Sclerotinia sclerotiorum (local isolate from Argentine) secreted two extracellular PGs (exo-PG-I and endo-PG-II) when the fungus was grown in liquid medium with polygalacturonic acid as the sole carbon source. Our results are different from others,

where more complex patterns in cultures are reported (Ryan 1994, Cervone 1986, Favaron *et al.* 1992, Riou 1993). It is possible that, like other pathogenic fungi, different isolates of *Sclerotinia sclerotiorum* produce different patterns of PG isoenzymes. In this paper we describe the purification and characterization of the endo-PG.

The production of endo-PG by *S. sclerotiorum* was induced by 0.5 % polygalacturonic acid in Maxwell medium. In this medium, PG activity reached a plateau after 7 d of culture. Like PGs produced by some other fungi, PGs of *S. sclerotiorum* were suppressed by the presence of 2 % glucose reported as non-inductive substrate (Fig. 1).

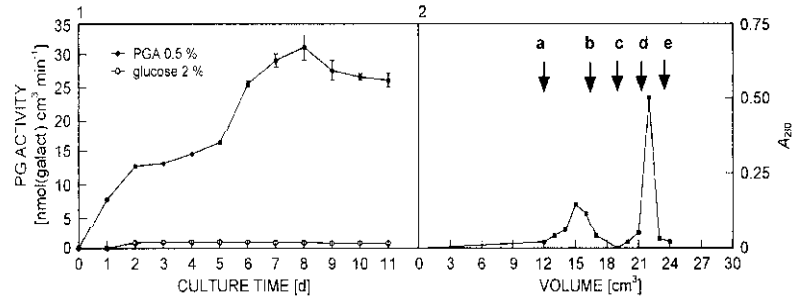

Fig. 1. Time-course of PG activity in the culture medium of *Sclerotinia sclerotiorum* with PGA or glucose as carbon source (means of two experiments ± SD).

Fig. 2. Elution of PG enzyme from *Superose 12* column (absorbance at 280 nm). The arrows indicate elution volume of molecular mass markers. *a* - aldolase (158 kDa), *b* - bovine serum albumine (66 kDa), *c* - ovalbumin (48 kDa), *d* - chymotrypsin (25 kDa), and *e* - ribonuclease (13.7 kDa).

The PG II purification was carried out as indicated in Materials and methods. The purified PG-II had a molecular mass of approximately 18 kDa estimated by gel filtration (Fig. 2). The isoelectric point (pI) of the purified PG-II determined by IEF was approximately 8 (not shown). Like other fungal PGs, *e.g.* from *Gaeumannomyces graminis*, *Geotrichum candidum* and *Botrytis cinerea* (Keon 1987), PG-II possesses positive charge at physiological pHs, and so it would bind to negatively charged pectins of plant cell wall and could degrade them. These results

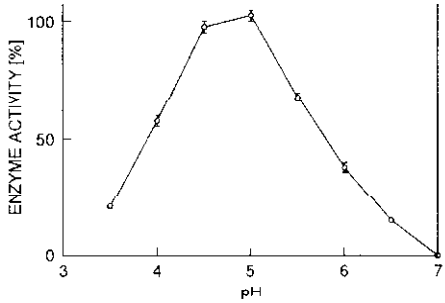

Fig. 3. Dependence of activity of the purified endo-PG on pH. Means of 2 determinations.

would suggest that PG-II remain localized near the area of infection, playing a key role in early stages of infection. Studies are in progress to test this hypothesis and in particular the interaction of PG-II with a polygalacturonase inhibiting protein which has been detected in sunflower (unpublished).

The enzyme is most active at pH 4.5 - 5.0 and it was almost inactive above pH 6 or below 3 (Fig. 3). The purified PG-II had no detectable pectate lyase activity as measured by the method described by Lee and West (Lee and West 1981). PGA was incubated for different times with the enzyme. The oligomers released were analysed by Mono Q anion exchange column as described in Materials and Methods. Fig. 4 shows that PG II was an endo-PG, inasmuch as there was no evidence (not even after exhaustive treatment of polygalacturonic acid with the enzyme) for the production of monomeric D-galacturonic acid as it would be expected from an exo enzyme.

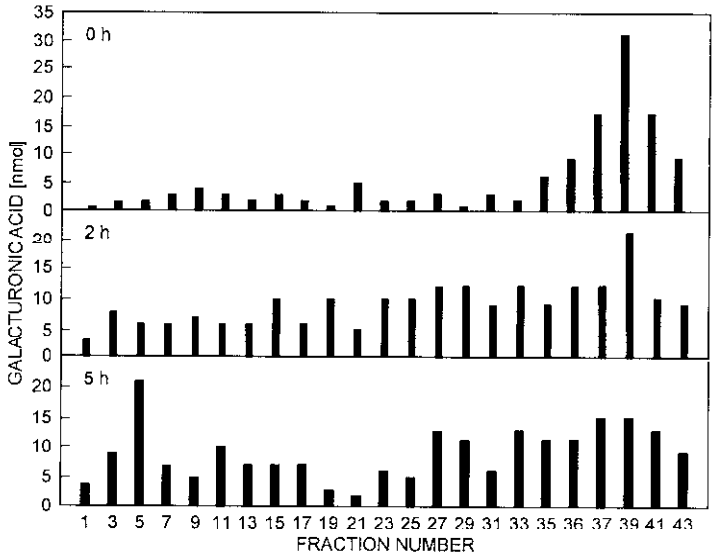

Fig. 4. Size distribution of products released from PGA by PG-II after 0-, 2- and 5-h incubation.

Taking into account that polygalacturonic acid is found naturally in plant cell wall as its partially methylated derivative (pectin), the catalytic activity of PG-II with pectin as the substrate was examined. The initial reaction rate was essentially the same with polygalacturonic acid or pectin as substrates (Fig. 5). The K_m value of the PG-II for polygalacturonic acid was determined from the initial reaction rates with substrate concentrations ranging from 0.011 % (m/v) to 0.4 % (m/v). The kinetic studies suggest that the reaction rate for *S. sclerotiorum* PG-II depends on substrate concentration. The K_m for the polygalacturonic acid in terms of polymer concentration can be estimated to be about 200 μM, and the V_{max} was approximately 40 μmol(reducing group) mg^{-1}(protein) min^{-1}, estimated with a Lineweaver-Burk plot (not shown). The results obtained were very similar to those of *Botrytis cinerea* PG (Yao 1995).

Although many PGs from phytopathogenic fungi have been purified and characterized, the results we report here suggest that the local isolate of *S. sclerotiorum* produces only one endo-PG that would be characterized by localized

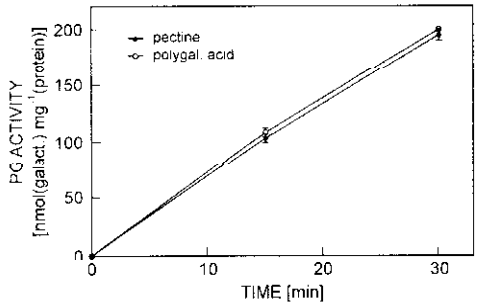

Fig. 5. PG-II polygalacturonase activities with PGA and pectin as substrates. Means of two experiments ± SD.

wall degradation This simplicity will be advantageous for isolating the PG-encoding gene and its subsequent molecular cloning and evaluation of its importance during pathogenicity. Future research should be aimed to establish correlations between sunflower PG inhibitor levels, effects of PG inhibitor on PG-II activity *in vitro*, and development of PG-II activity in lesions of sunflower lines with different susceptibility to *S. sclerotiorum*.

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