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Research Paper

Production, characterization, and identification using proteomic tools of a polygalacturonase from *Fusarium graminearum*

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Since enzymatic degradation is a mechanism or component of the aggressiveness of a pathogen, enzymatic activities from a *Fusarium graminearum* isolate obtained from infected wheat spikes of Argentina Pampa region were studied in order to understand the disease progression, tending to help disease control. In particular, the significance of the study of polygalacturonase activity is based on that such activity is produced in the early stages of infection on the host, suggesting a crucial role in the establishment of disease. In this sense, polygalacturonase activity produced by this microorganism has been purified 375 times from 2-day-old culture filtrates by gel filtration and ion-exchange chromatography successively. The purified sample showed two protein bands in sodium dodecyl sulfate–polyacrylamide gels, with a molecular mass of 40 and 55 kDa. The protein bands were identified as an endopolygalacturonase and as a serine carboxypeptidase of *F. graminearum*, respectively, by peptide mass fingerprinting (matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF/TOF) fragment ion analysis). The pattern of substrate degradation analyzed by thin layer chromatography confirmed the mode of action of the enzyme as an endopolygalacturonase. High activity of the polygalacturonase against polygalacturonic acid was observed between 4 and 6 of pH, and between 30 and 50 °C, being 5 and 50 °C the optimum pH and temperature, respectively. The enzyme was fully stable at pH 5 for 120 min and 30 °C and sensible to the presence of some metal ions. This information would contribute to understand the most favorable environmental conditions for establishment of the disease.

Abbreviations: ADG – digalacturonic acid; **AGA** – galacturonic acid; **ATG** – trigalacturonic acid; **CWDE** – cell wall-degrading enzymes; **EDTA** – ethylenediaminetetraacetic acid; **FPLC** – fast protein liquid chromatography; **IEF** – isoelectrofocusing; **LMW** – low-molecular-weight markers; **MALDI TOF/TOF** – matrix-assisted laser desorption/ionization time-of-flight; **MS** – mass spectrometry; **PG** – polygalacturonase; **PMF** – peptide mass fingerprinting; **SDS–PAGE** – sodium dodecyl sulfate–polyacrylamide gel electrophoresis; **SNA** – synthetic nutrient agar; **TFA** – trifluoroacetic acid; **TLC** – thin layer chromatography

Keywords: Endopolygalacturonase / *Fusarium graminearum* / Pectinases / Peptide mass fingerprinting / Wheat

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Introduction

Plant pathogenic fungi produce extracellular enzymes, which can degrade the cell wall components of plants (CWDE: cell wall-degrading enzymes). Research on *Fusarium* spp. reached conclusive evidence on the role and importance of these enzymes in the infection process [1, 2]. A reduced secretion of CWDE might retard

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both, the growth of the fungus on the host surface and its infection process, giving the host more time for a defensive response [1]. Among the CWDE early secreted by fungal pathogens during infection, pectic enzymes, and in particular endopolygalacturonases (PG), are essential in the process of virulence [3–5]. They contribute to pathogenicity and/or virulence of several phytopathogenic fungi by degrading the pectin component of the cell wall and of middle lamella [2, 6, 7], producing modifications enabling the accessibility of cell wall components for degradation by other enzymes [8]. Several studies have concluded that pectinases are virulence factors for phytopathogenic *Fusarium* spp., which are secreted during the early stages of infection [9, 10]. Ferrari *et al.* [10] suggest that endopolygalacturonase probably plays a relevant role in *Fusarium graminearum* infection on wheat, based on the analysis of transgenic expression of polygalacturonase inhibiting proteins. Another group of enzymes that act together to CWDE to invade the host, are the proteinases, degrading the structural proteins of the cell wall [11]. In addition, these enzymes are associated with the loss of grain quality by modifying their storage protein [12, 13].

F. graminearum is the predominant associated pathogen to an important wheat disease worldwide, such as Fusarium Head Blight. This disease produces high economic losses due to yield and quality reduction, as well as contamination by mycotoxins in infected grains [14, 15].

The enzymatic study conducted in this research was based on the fact that detection, purification/identification using proteomic tools and characterization of polygalacturonase can contribute to the interpretation of both, the enzymatic mechanisms and role in establishment of infection on wheat.

Materials and methods

Biological material and culture conditions

An enzymatic screening was conducted from *F. graminearum* isolates obtained from wheat spikes from Argentina Pampas region [16]. Based on this data, the isolate number 1 was selected for this study, because of its high PG activity. Stock cultures (monosporic isolate) were kept at 4 °C on 2% w/v synthetic nutrient agar (SNA) slants under a mineral oil layer. Cultures were conducted in darkness for 15 days in 125-ml Erlenmeyers flasks containing 25 ml of a modified Czapek-Dox medium [7] with 0.25% w/v glucose, 0.125% w/v citrus pectin, and 0.125% w/v commercial oat bran as carbon sources and/or enzyme inducers, at 28 °C and 200 rpm. The inoculum was prepared from 5-mm plugs cut out from the margin of a 5-day-old colony growing on Petri dishes (2% w/v potato-agar) at 26 °C. The whole content of each Erlenmeyer flask was withdrawn and analyzed for enzyme activity.

Preparation of crude enzyme extracts

Mycelium of *F. graminearum* isolate was separated by centrifugation (5000*g*) from 2-day-old cultures, grown in 1000-ml Erlenmeyer flask. The supernatant was 100-fold concentrated by evaporation at 30 °C under reduced pressure, then desalted with a pre-packed Sephadex G-25 mini column (Amersham) and stored at −70 °C until use.

Polygalacturonase activity assay

Polygalacturonase (PG) activity was determined at 40 °C using 0.1% polygalacturonic acid in 50 mM acetate buffer pH 5.0 as substrate. The enzymatic activity was determined by measuring the liberation of reducing groups by Somogyi-Nelson method [17]. One enzymatic unit was defined as the amount of necessary enzyme to release 1 μmol of galacturonic acid (AGA) per minute under the above mentioned reaction conditions. Protein content was determined by the Bradford [18] method.

Chromatographic purification

All chromatographic steps were carried out on an Amersham ÄKTA FPLC-U900 system (General Electric). Crude enzyme extracts were applied to a Sephacryl S-100 gel-filtration column (XK 16/100, General Electric Little Chalfont, UK) pre-equilibrated with acetate buffer (20 mM, pH 5.0 with 0.15 M NaCl), and eluted isocratically with the same buffer at a flow rate of 0.5 ml min^{-1}. Fractions with PG activity were pooled, concentrated by lyophilization, resuspended in the same buffer, and rechromatographed onto a Sepharose SP (XK 26/20, General Electric) cation exchange column, pre-equilibrated with acetate buffer (20 mM, pH 4.0). Proteins were eluted with a linear gradient of NaCl (0–0.4 M) in acetate buffer at a flow rate of 1 ml min^{-1}. Fractions with PG activity were pooled, concentrated by lyophilization and resuspended in the same buffer. The enzyme preparation was stored at −70 °C and used for further characterization.

Gel electrophoresis

After a prior desalting step, the molecular size and purity of the protein were estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12% w/v gel calibrated with low-molecular-weight markers (LMW Kit, General Electric) according to Laemmli [19]. The protein bands were silver stained [20].

Protein identification

Protein bands of purified sample obtained by SDS–PAGE were cut from the gel and subjected to tryptic digestion. Peptide mass fingerprinting (PMF) of protein selected spots was carried out by in-gel trypsin treatment (sequencing-grade Promega) at 37 °C overnight. Peptides were extracted from the gels by using 60% acetonitrile in 0.2% TFA, concentrated by vacuum drying and desalted by using C_{18} reverse phase micro-columns (OMIX Pipette tips, Varian). Peptide elution from micro-column was performed directly into the mass spectrometer sample plate with 3 μl of matrix solution (α-cyano-4-hydroxycinnamic acid in 60% aqueous acetonitrile containing 0.2% TFA). Mass spectra of digestion mixtures were acquired in a 4800 MALDI-TOF/TOF instrument (Applied Biosystem) in reflector mode and were externally calibrated using a mixture of peptide standards [21]. Collision-induced dissociation MS/MS experiments of selected peptides were performed. Proteins were identified by NCB Inr database search with peptide *m/z* values using MASCOT search tool (URL http://www.matrixscience.com) for identification of tryptic maps.

Substrate degradation pattern

Partially purified PG enzyme was incubated at 37 °C with 0.4% polygalacturonic acid in 50 mM acetate buffer, pH 4.0. Samples were taken at different times: 0, 0.5, 1, 3, and 30 h, incubated at 100 °C for 5 min and reducing sugars were measured by Somogyi-Nelson method [17]. The mixtures of reaction were analyzed by thin layer chromatography (TLC), loading 2 μl of each sample and 2 μl of standards (1000 ppm) of AGA, digalacturonic acid (ADG), and trigalacturonic acid (ATG) on a silica gel plate (Merck, USA; TLC aluminum sheets 20 cm × 20 cm, Silica gel F_{254}). A mixture of *n*-butanol: acetic acid: water (9:4:7 v/v/v) was used as mobile phase. Plates were air-dried and products were revealed by spraying a solution of 3% (w/v) phosphomolybdic acid and 10% v/v sulfuric acid in ethanol and heating for 10 min at 90 °C.

Effect of pH on enzyme activity and stability

PG activity was measured under standard conditions at different pH values (from 2.0 to 8.0) using a borate/citrate/phosphate buffer (50 mM). The pH effect on the stability of the enzyme was evaluated at various pH values (from 3.0 to 6.0) by incubating enzyme solutions at 40 °C for 120 min. Samples were taken at regular intervals and activity was measured as previously described.

Effect of temperature on enzyme activity and stability

PG activity was measured at pH 5.0 at different reaction temperatures (from 20 to 80 °C). In order to determine the temperature effect on the stability of enzyme preparations at the optimum pH, enzyme solutions were kept for 120 min at various temperatures (from 30 to 50 °C). Samples were taken at regular intervals and then the activity was measured as previously described.

Effect of metal ions on enzyme activity

PG activity was measured as previously described after adding different metal ions: (10 mM) and EDTA (2 mM) to the reaction mixture. The following salts were used: $HgCl_2$, $CuSO_2$, $CaCl_2$, $BaCl_2$, $CoCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, and $NaCl_2$.

Results

Time course of polygalacturonase activity produced by *F. graminearum* isolate employed in this work, was followed daily in culture supernatants for 15 days. The highest PG activity was found between the 2nd and 3rd day of culture time from which these values gradually decreased (Fig. 1).

Polygalacturonase was purified 375 times with a specific activity of 5.41 U mg^{-1} and a recovery of 6.4% from 2-day-old culture filtrate by two chromatographic steps purification. The first step of purification consisted in a gel filtration, which allowed removal of pigments, and the second step of purification was represented by an ion-exchange chromatography (Fig. 2), both carried out in an FPLC. The electrophoretic profile under denaturing conditions of each purification step is shown in Fig. 3. Two protein bands (with molecular mass of 40 and 55 kDa) were identified after ion exchange chromatography; these bands (Fig. 3a and b) were cut from the gel, subjected to tryptic digestion and analyzed by peptide mapping by MALDI-TOF MS. The 40 kDa protein was

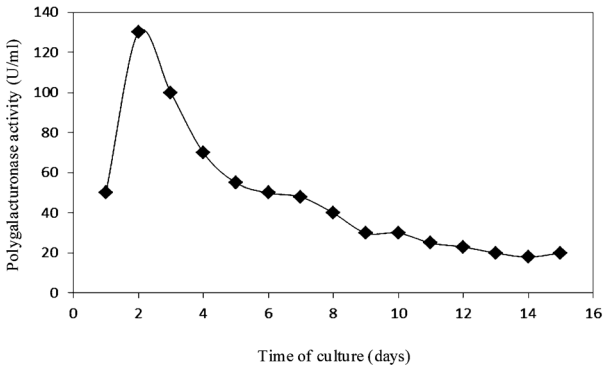

Figure 1. Polygalacturonase activity produced by *Fusarium graminearum* as a function of the time of culture. Values are means of six replicates.

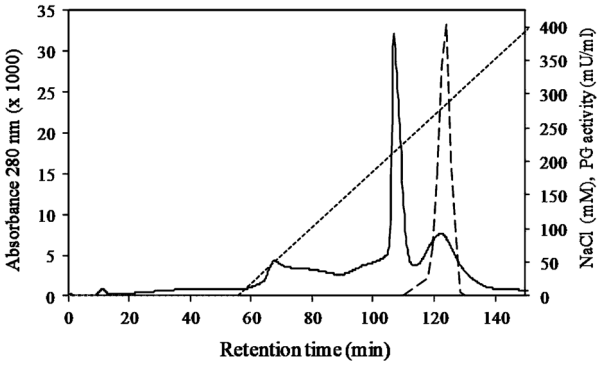

Figure 2. Ion-exchange chromatography. Elution profile of protein (absorbance at 280 nm: solid line) and polygalacturonase activity (broken line) on a Sepharose SP cation-exchange column in a linear gradient of NaCl (dotted line).

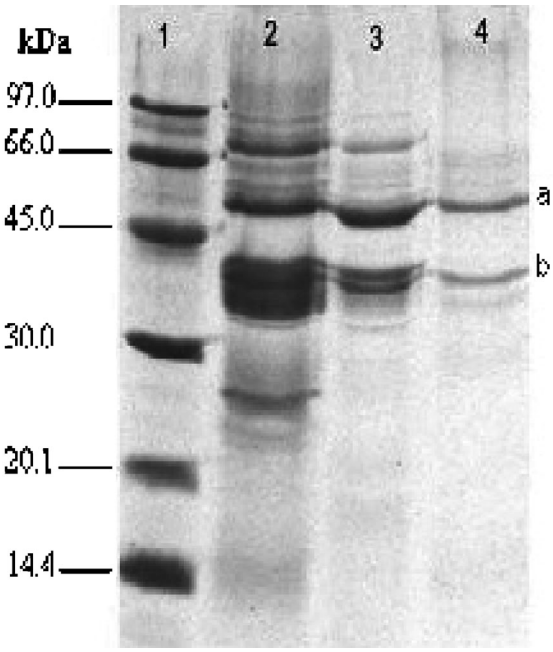

Figure 3. SDS–PAGE. Line 1: protein calibration kit (BioRad 14,500–90,000 Da), line 2: crude extract, line 3: eluate of gel filtration chromatography, line 4: eluate of ion exchange chromatography. (a) Serine carboxy-peptidase, (b) endo-polygalacturonase.

identified as a hypothetical protein FG11011.1 (I1S2L3, UniProtKB/TrEMBL) of *F. graminearum*. This protein corresponds to a endopolygalacturonase enzyme which, according to the Fungal Secretome Knowledge Base (http://www.proteomics.ysu.edu/secretomes/fungi.php) [22] is a secreted protein whose first 17 amino acid residues encode to the signal sequence as predicted using

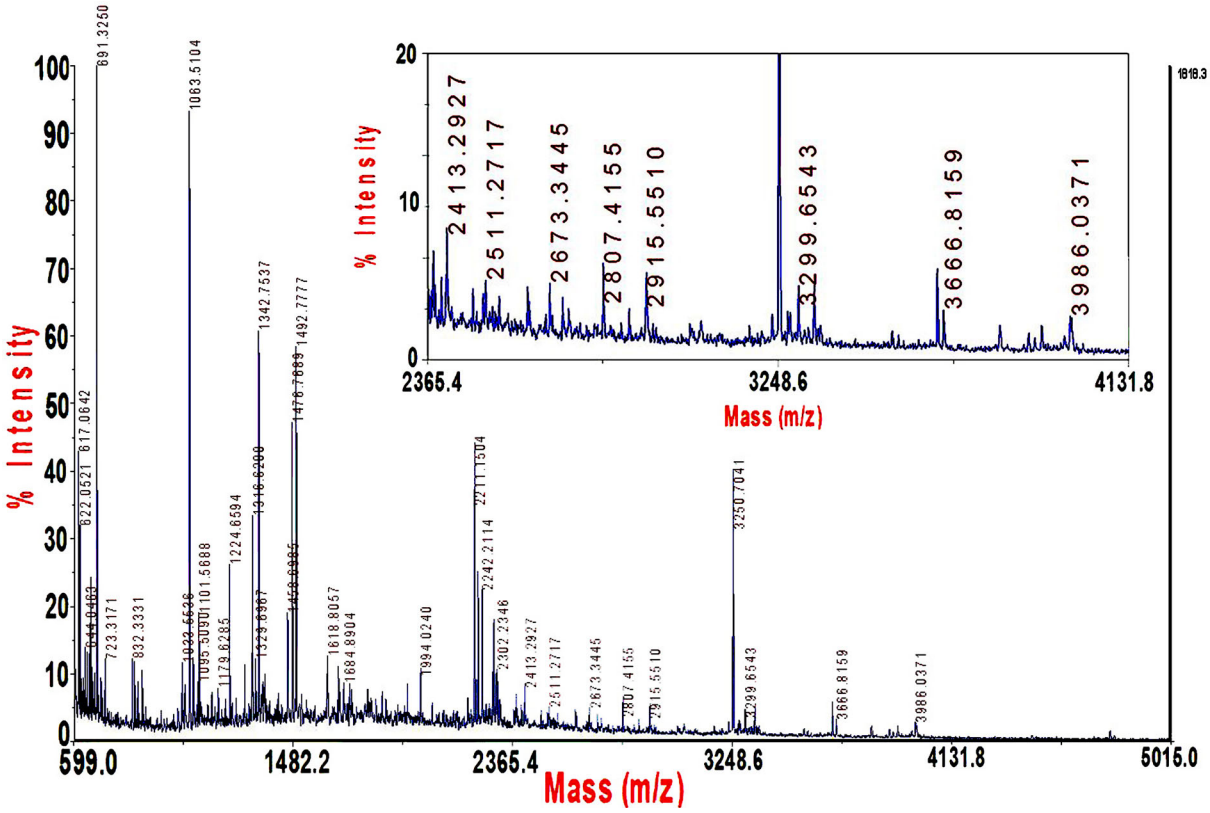

Figure 4. Mass spectrum obtained by MALDI-TOF-MS of 40 kDa protein.

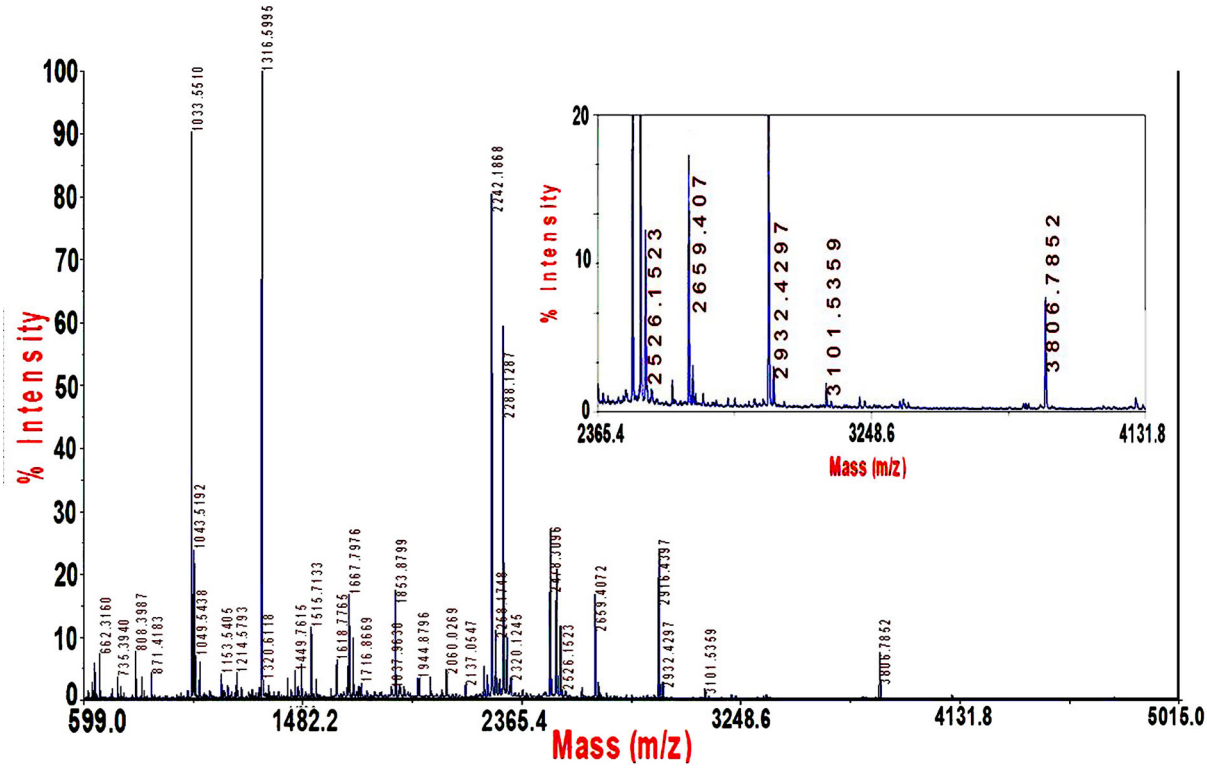

Figure 5. Mass spectrum obtained by MALDI-TOF-MS of. 55 kDa protein.

the SignalP 3.0 algorithm (http://www.cbs.dtu.dk/services/SignalP/) [23] (Fig. 4). The 55 kDa protein was identified as a hypothetical protein FG04527 serine carboxypeptidase of *F. graminearum*, by statistically significant similarities (Fig. 5).

The purified polygalacturonase enzyme exhibited high activity (more than 80% of maximum activity) between 30 and 50 °C (range tested: 20–80 °C, Fig. 6) and between pH 4 and 6 (range tested: 2–8, Fig. 7), being 5 and 50 °C the optimum pH and temperature, respectively. The enzyme was fully stable at pH 5 for 120 min and 30 °C. When different metal ions were tested on PG activity, increasing inhibition was observed in the following order: Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+}, Hg^{2+}, while it was not inhibited by the presence of Na^{2+} ions (Table 1).

As shown in Fig. 8, according to the substrate fragmentation pattern analyzed by TLC from the

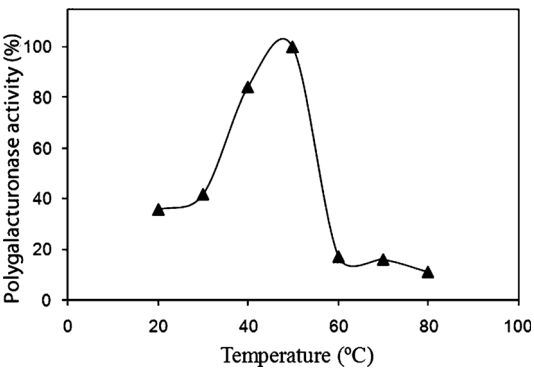

Figure 6. Effect of temperature on polygalacturonase activity from *F. graminearum*.

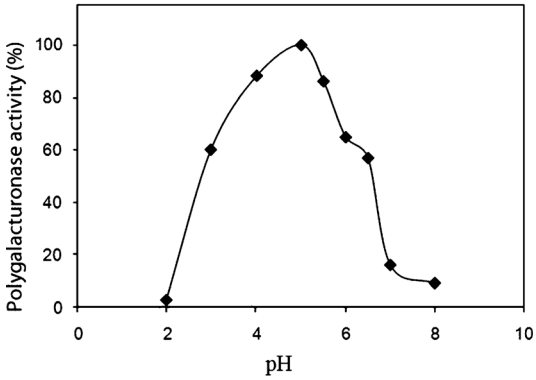

Figure 7. Effect of pH on polygalacturonase activity from *F. graminearum*.

Table 1. Effect of metal ions on polygalacturonase activity from *F. graminearum*.

Metal ions (10 mM)	Relative activity (%)
Control	100.0
Na^{2+}	101.0
Mg^{2+}	95.0
Mn^{2+}	47.0
Co^{2+}	45.0
Zn^{2+}	21.0
Cu^{2+}	18.0
Ca^{2+}	16.0
Ba^{2+}	10.0
Hg^{2+}	6.0
EDTA (2 mM)	56.0

products resulting of the PG activity on polygalacturonic acid at different times using acid mono-, di-, and tri-galacturonic patterns, the purified PG activity was found to be an endopolygalacturonase.

Discussion

It is well known the close correlation between the production of enzymes by *Fusarium* spp. with its virulence, symptoms of disease, or degree of damage observed on the host [24, 25]. Several genes of *F. graminearum* have been studied in terms of the infection process of wheat, allowing elucidating the role of enzymes in this course. The creation of constructs with disrupted genes has enabled the analysis of the secretor pathways of these enzymes and thus determining their function [26–30].

In the present work, a polygalacturonase found in early stages of cultivation of *F. graminearum* was studied.

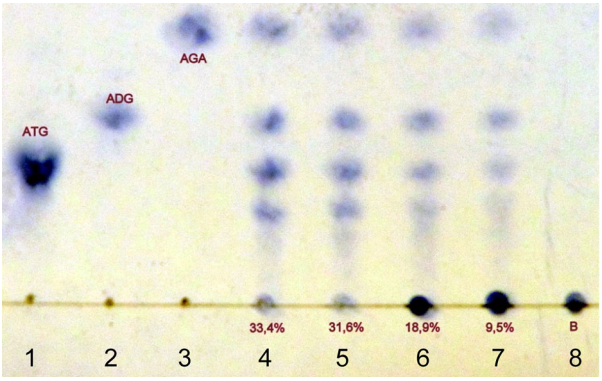

Figure 8. Fragmentation pattern of the purified endo-polygalacturonase by TLC. Patterns: trigalacturonic acid, ADG, and galacturonic acid (lines 1–3, respectively). Products resulting from the PG activity on polygalacturonic acid at 30, 3, 1, and 0.5 h (lines 4–7, respectively and 33.4–9.5% indicates fragmentation percentage). Blank (line 8).

The presence of this enzyme would be related to the location of the pectin in the cell wall, so its production may play a crucial role in the establishment of disease. The endopolygalacturonase identified in the present report resulted of interest since among pectinases, the endo-polygalacturonase is considered a relevant enzyme due to its mechanism of action in the degradation of pectin, which produces strong depolymerization. The data observed in the characterization of polygalacturonase activity in *F. graminearum* isolate, regarding to optimum values of pH and temperature, and the stability of these parameters, resembles at data obtained with other filamentous fungi, like *Fusarium oxysporum*, *Fusarium culmorum*, *Aspergillus* sp., and *Trichoderma* sp. [3, 6]. Besides, the polygalacturonase activity was sensitive to the presence of EDTA and some metal ions. Further, these results agree with those reported by Tomassini *et al.* [31] who reported that PG encoding genes in *F. graminearum* were maximum expressed at 24 h after wheat spikelets infection, and also describes a polygalacturonase with some similar biochemical characteristics. This information would contribute to understand the most favorable environmental conditions for establishment of the disease.

On the other hand, the protein identified as a serine carboxypeptidase belongs to a protein family widely distributed in fungi, and particularly abundant in filamentous fungi [32]. According to several researches, *Fusarium* peptidases, together with other hydrolytic enzymes and toxins, are strongly involved in the fungal colonization of grains [12, 26, 33, 34].

The high intraspecific genetic variability presumed among *F. graminearum* isolates, would involve differential behavior in terms of aggressiveness against host. Therefore, since enzymatic degradation is a mechanism or component of the aggressiveness of a pathogen, our study is helpful to understand the disease progression, tending to help disease control.

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Conflict of interest statement

There is not any kind of "conflict of interests," financial or commercial, with any trademark mentioned in our

manuscript, competitive interest, or secondary interest that could influenced our research. This declaration is carried out by all the authors of the work presented.

References

[1] Jenczmionka, N.J., Schafer, W., 2005. The Gpmk1 MAP kinase of *Fusarium graminearum* regulates the induction of specific secreted enzymes. Curr. Genet., **47**, 29–36.

[2] Wanyoike, W.M., Kang, Z., Buchenauer, H., 2002. Importance of cell wall degrading enzymes produced by *Fusarium graminearum* during infection of wheat head. Eur. J. Plant Pathol., **108**, 803–810.

[3] Martínez, M.J., Alconada, T.M., Guillén, F., Vázquez, C., Reyes, F., 1991. Pectic activities from *Fusarium oxysporum* f. sp. *Melonis*: purification and characterization of an exopolygalacturonase. FEMS Microbiol. Lett., **81**, 145–150.

[4] Isshiki, A., Akimitsu, K., Yamamoto, M., Yamamoto, H., 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. Mol. Plant Microbe Interact., **14**, 749–757.

[5] Oeser, B., Heidrich, P.M., Müller, U., Tudzynski, P., Tenberge, K.B., 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. Fungal Genet. Biol., **36**, 176–186.

[6] Kikot, G., Hours, R., Alconada, T., 2009. Contribution of cell wall degrading enzymes to pathogenesis of *Fusarium graminearum*: a review. J. Basic Microbiol., **49**, 231–241.

[7] Kikot, G., Hours, R., Alconada, T., 2010. Extracellular enzymes of *Fusarium graminearum* isolates. Braz. Arch. Biol. Technol., **53**, 779–783.

[8] Panda, T., Nair, S.R., Kumar, P., 2004. Regulation of synthesis of the pectolytic enzymes of *Aspergillus niger*. Enzyme Microb. Technol., **34**, 466–473.

[9] García-Maceira, F.I., Di Pietro, A.D., Huertas-González, M.D., Ruiz-Roldán, M.C., Roncero, M.I., 2001. Molecular characterization of an endopolygalacturonase from *Fusarium oxysporum* expressed during early stages of infection. Appl. Environ. Microbiol., **67**, 2191–2196.

[10] Ferrari, S., Sella, L., Janni, M., De Lorenzo, G. et al., 2012. Transgenic expression of polygalacturonase-inhibiting proteins in *Arabidopsis* and wheat increases resistance to the flower pathogen *Fusarium graminearum*. Plant Biol., **14**, 31–38.

[11] Walton, J.D., 1994. Deconstructing the cell wall. Plant Physiol., **104**, 1113–1118.

[12] Nightingale, M.J., Marchylo, B.A., Clear, R.M., Dexter, J.E., Preston, K.R., 1999. Fusarium Head Blight: effect of fungal proteases on wheat storage proteins. Cereal Chem., **76**, 150–158.

[13] Barneix, A.J., 2007. Physiology and biochemistry of source-regulated protein accumulation in the wheat grain. J. Plant Physiol., **164**, 581–590.

[14] Kikot, G., Moschini, R., Consolo, F., Rojo, R. et al., 2011. Occurrence of *Fusarium graminearum* from wheat in relation to climatic models in Argentina Pampas Region. Mycopathologia, **171**, 139–149.

[15] Çepni, E., Tunali, B., Gürel, F., 2013. Genetic diversity and mating types of *Fusarium culmorum* and *Fusarium graminearum* originating from different agro-ecological regions in Turkey. J. Basic Microbiol., **53**(8), 686–694.

[16] Kikot, G., 2012. Caracterización bioquímica, fenotípica y molecular de aislamientos de *Fusarium graminearum* provenientes de la región pampeana en relación a la patogenicidad, *Ph.D. Thesis*.

[17] Somogyi, N., 1952. Notes on sugar determination. J. Biol. Chem., **195**, 19–23.

[18] Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Ann. Clin. Biochem., **72**, 248–254.

[19] Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680–685.

[20] Morrissey, J.H., 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem., **117**, 307–310.

[21] Patterson, S.D., Aebersold, R., 1995. Mass-spectrometric approaches for the identification of gel-separated proteins. Electrophoresis, **16**, 1791–1814.

[22] Lum, G., Min, X.J., 2011. FunSecKB: the Fungal Secretome Knowledge Base. Database, **2011**, 1–10

[23] Brendtsen, J.D., Nielsen, H., von Heijne, J., Brunak, S., 2004. Improved prediction of signal peptides: Signal P 3.0. J. Mol. Biol., **340**, 783–795.

[24] ten Have, A., Tenberge, K.B., Benen, J.A.E., Tudzynski, P. et al., 2002. The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens, in: Kempken, F. (Ed.), The Mycota. XI. Agricultural Application, Springer-Verlag, Berlin/Heidelberg, 341–358.

[25] Lang, C., Dörnenburg, H., 2000. Perspectives in the biological function and the technological application of polygalacturonases. Appl. Microbiol. Biotechnol., **53**, 366–375.

[26] Voigt, C.A., Schafer, W., Salomon, S., 2005. A secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals. Plant J., **42**, 364–375.

[27] Hou, Z., Xue, C., Peng, Y., Katan, T. et al., 2002. A mitogen-activated protein kinase gene (MGVI) in *Fusarium graminearum* is required for female fertility, heterokaryon formation and plant infection. Mol. Plant Microbe Interact., **15**, 1119–1127.

[28] Jenczmionka, N.J., Maier, F.J., Lösch, A.P., Schäfer, F.J., 2003. Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main causal agent of the head blight disease of wheat, are regulated by the MAP kinase gpmk1. Curr. Genet., **43**, 87–95.

[29] Lu, S.W., Kroken, S., Lee, B.N., Robbertse, B. et al., 2003. A novel class of gene controlling virulence in plant pathogenic ascomycete fungi. Proc. Nat. Acad. Sci. USA, **100**, 5980–5985.

[30] Seong, K., Hou, Z., Tracy, M., Kistler, H.C., Xu, J.-R., 2005. Random insertional mutagenesis identifies genes

associated with virulence in the wheat scab fungus *Fusarium graminearum*. Phytopathology, **95**, 744–750.

[31] Tomassini, A., Sella, L., Raiola, A., D'Ovidio, R., Favaron, F., 2009. Characterization and expression of *Fusarium graminearum* endo-polygalacturonases *in vitro* and during wheat infection. Plant Pathol., **58**, 556–564.

[32] Blinkovsky, A.M., Byun, T., Brown, K.M., Golightly, E.J., 1999. Purification, characterization, and heterologous expression in *Fusarium venenatum* of a novel serine carboxypeptidase from *Aspergillus oryzae*. Appl. Environ. Microbiol., **65**, 3298–3303.

[33] Schwarz, P.B., Jones, B.L., 2002. Enzymes associated with *Fusarium* infection of barley. J. Am. Acad. Soc. Brew. Chem., **60**, 130–134.

[34] Pekkarinen, A.I., Longstaff, C., Jones, B.L., 2007. Kinetics of the inhibition of *Fusarium* serine proteinases by barley (*Hordeum vulgare* L.) inhibitors. J. Agric. Food Chem., **55**, 2736–2742.