Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol
DOI: 10.1159/000460296

Received: June 16, 2016
Accepted: February 8, 2017
Published online: ■■■

Purification and Biochemical and Kinetic Properties of an Endo-Polygalacturonase from the Industrial Fungus *Aspergillus sojae*

Dante Fratebianchi Ivana Alejandra Cavello Sebastián Fernando Cavalitto

Research and Development Center for Industrial Fermentations, CINDEFI (CONICET – La Plata, UNLP), La Plata, Argentina

Please confirm that authors' names and initials have been identified correctly.
Please confirm the phrase `at any case', which occurs in the Introduction.

Keywords
Endo-polygalacturonase · Enzyme purification · Biochemical characterization · Kinetic properties · *Aspergillus sojae*

Abstract
An endo-polygalacturonase secreted by *Aspergillus sojae* was characterized after being purified to homogeneity from submerged cultures with orange peel as the sole carbon source by gel filtration and ion-exchange chromatographies. According to SDS-PAGE and analytical isoelectric focusing analyses, the enzyme presents a molecular weight of 47 kDa and pI value of 4.2. This enzyme exhibits considerable stability under highly acidic to neutral conditions (pH 1.5–6.5) and presents a half-life of 2 h at 50°C. Besides its activity towards pectin and polygalacturonic acid, the enzyme displays pectin-releasing activity, acting best in a pH range of 3.3–5.0. Thin-layer chromatographic analysis revealed that tri-galacturonate is the main enzymatic end product of polygalacturonic acid hydrolysis, indicating that it is an endo-polygalacturonase. The enzyme exhibits Michaelis-Menten kinetics, with K_M and V_{MAX} values of 0.134 mg/mL and 9.6 µmol/mg/min, respectively, and remained stable and active in the presence of SO_2, ethanol, and various cations assayed except Hg^{2+}.

Introduction

Pectinases comprise a highly diverse group of biocatalysts responsible for the enzymatic degradation of pectic substances, whose function resides in providing integrity to the plant tissues. Among pectinases, the most thoroughly studied subgroup is involved in the degradation of the so-called smooth region of the pectin backbone (homogalacturonan), which includes esterases and depolymerases. These enzymes have been widely used within the fruit and vegetable processing industry for the extraction and clarification of fruit juice, and mash treatment, and within the wine industry for clarification of white wine must and to improve color extraction during red wine elaboration [Grassin and Fauquembergue, 2009; van Rensburg and Pretorius, 2000]. The intrinsic acidity of fruit juices and wines requires the use of acidic pectinases, most of which are commercially produced from fungal sources.

Polygalacturonases (PGs) are depolymerizing enzymes that degrade homogalacturonan by hydrolysis of α(1–4) glycosidic bonds between galacturonate residues. Tailor-made enzymatic preparations for several industrial purposes tend to constitute of a complex mix of polysaccharide-degrading enzymes in which PGs are usually present.

KARGER

E-Mail karger@karger.com
www.karger.com/mmb

Sebastián Fernando Cavalitto
Research and Development Center for Industrial Fermentations
CINDEFI (CONICET – La Plata, UNLP). Calle 47 y 115
La Plata B1900ASH (Argentina)
E-Mail cavalitto@quimica.unlp.edu.ar

Table 1. The main steps involved in the purification of *A. sojae* polygalacturonase

Purification step	Volume, mL	Total protein, mg	Total activity, U_{PG}	Specific activity, U_{PG}/mg	Yield, %	Purification (fold)
Crude extract	400	14.73	12,593	854.7	100	1
Q Sepharose	200	4.07	10,657	2,619	84.6	3.06
SP Sepharose	200	1.18	9,147	7,738	72.6	9.05
Superdex 75	30	0.41	5,370	13,023	42.6	15.2

Aspergillus sojae is a koji mold that has been extensively used in traditional Asian food fermentation processes, such as the production of miso and soya sauce [Bourdichon et al., 2012]. Several studies have been performed regarding PG production by *A. sojae* growing on complex as well as simple sugar-containing substrates [Fratebianchi et al., 2017; Gögus et al., 2006]. Nevertheless, very few were aimed at characterizing the enzymes responsible for this activity, having worked with crude or semipurified extracts at any case [Dogan and Tari, 2008; Tari et al., 2008]. The study of the properties of *A. sojae* PG(s) is essential to determine the extent of its potential industrial applications, and to assess the feasibility for large-scale processes.

As pectin is probably one of the most complex macromolecules in nature, characterization of pectin-degrading activities in a crude extract has the drawback of being unable to determine whether the effects are caused by a single enzyme or by a multienzymatic consortium. Besides, crude extracts may contain substances that can act as enzymatic inhibitors and have to be eliminated before performing a proper analysis. Therefore, in order to unequivocally characterize and study the properties of pectinases, prior purification is highly recommended. In the present investigation, we describe the purification of an endo-PG secreted by *A. sojae* ATCC 20235 when grown in submerged cultures with orange peel as the substrate, and proceed to characterize the isolated enzyme in terms of its biochemical and kinetic properties.

Results and Discussion

Pectinolytic Production Profile of A. sojae

Pectin-depolymerizing activities (PG, endo-PG, and pectin lyase, PL) were measured during the growth of *A. sojae* in a culture medium which consisted of ground orange peel and ammonium sulfate. The time course of the production of PG and endopectinase (endoP) activities shows that both activities increased in parallel throughout the culture and reached maximum values from 168 h onwards; enzyme purification steps were therefore performed from 7-day-old cultures (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000460296 for all online suppl. material). Similar kinetics of PG production by filamentous fungi in shake-flask cultures have been reported [Galiotou-Panayotou et al., 1997; Malvessi and Silveira, 2004]. In contrast, solid-state cultures of *A. sojae* on wheat bran and orange peel gave maximum PG activity after 120 h of incubation [Heerd et al., 2012].

As endoP activity is determined by a viscometric method, it reflects the total depolymerization activity potentially caused by both PL and PG enzymes [Gusakov et al., 2002]. However, no PL activity was detected during the cultivation, suggesting endoP and PG activities result from the production of at least 1 endo-PG enzyme. The viscometric method is time consuming and allows fewer simultaneous sample determinations compared to the reducing sugar method. Because of the above, and since the endoP/PG activity ratio was kept fairly constant along the culture, the PG activity assay was the method chosen to follow the purification process.

Enzyme Purification

A PG was purified to homogeneity from the supernatant of a 7-day-old culture of *A. sojae* by gel filtration and ion exchange chromatographies. The purification procedure is summarized in Table 1. In addition to PG activity, the starting crude extract presented weak pectinesterase (PE) activity. This concomitant activity along with other contaminants was successfully separated from the PG-active fraction by Q Sepharose followed by Sp Sepharose chromatographies. After a final polishing step on a Superdex 75 column, a homogeneous fraction showing PG activity was obtained with a 15.2-fold enrichment and a 42.6% recovery. Zymogram and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis revealed a single homogeneous band with activity towards polygalacturonic acid (PGA; Fig. 1a).

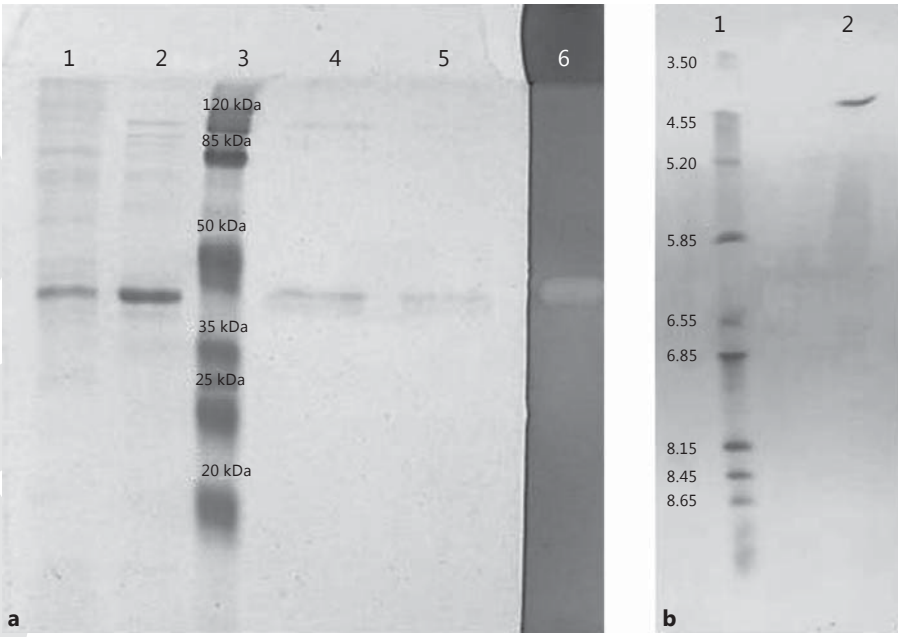

Fig. 1. a SDS-PAGE analysis of the purification process of *A. sojae* PG. Lane 1, crude extract; lane 2, sample obtained from Q Sepharose chromatography; lane 3, prestained protein molecular weight marker (20–120 kDa; Thermo Scientific, Rockford, IL, USA); lane 4, sample obtained from SP Sepharose chromatography; lane 5, sample obtained from Superdex 75 chromatography; lane 6, zymogram of the sample obtained from Superdex 75 chromatography. **b** Analytical IEF. Lane 1, broad range pI marker (GE); lane 2, purified PG.

Biochemical Characterization

The apparent molecular weight of *A. sojae* PG determined by SDS-PAGE was 47.0 ± 0.9 kDa. In another study, 2 different protein bands of 36 and 53 kDa were observed on a gel after an SDS-PAGE of a partially purified PG-active fraction produced by *A. sojae* on submerged cultures [Tari et al., 2008]. The small difference in molecular weight between this 53-kDa protein and the *A. sojae* PG presented here suggests that both might actually be the same molecule. Nevertheless, more than 1 pectinolytic enzyme can be produced by the same mold [Dinu et al., 2007; Semenova et al., 2003]. PGs secreted by a wide variety of microorganisms, such as *Fusarium graminearum* [Ortega et al., 2014], *Saccharomyces cerevisiae* [Gainvors et al., 2000], or *Streptomyces lydicus* [Jacob et al., 2008], present molecular mass values of 40, 42, and 43 kDa, respectively, ranging within the value found in this study.

Analytical isoelectric focusing (IEF) revealed a pI value of approximately 4.2 for the purified enzyme (Fig. 1b), which is lower than that reported of most microbial PGs [Jayani et al., 2005] although it fits into the pI range described for these enzymes.

The pH stability of *A. sojae* PG was studied within the pH range of 1.0–6.5. After 2 h of incubation, the purified enzyme retained more than 75% of its initial activity between the pH values of 2.0 and 6.5, and showed up to 55% residual activity within a very low pH interval (1.0–2.0; Fig. 2a). The purified PG was further incubated for 24 h in the pH range of 1.0–3.0, retaining 35% of its initial activity at pH 1.0 and more than 75% from pH 1.5 onwards. Fungal PGs tend to be stable under acidic conditions [Jayani et al., 2005], although stability studies are seldom performed at a pH of approximately 2.

Regarding the effect of temperature, the enzyme was fully thermostable up to 45°C, whereas 25% of the initial activity was retained after 210 min of incubation at 50°C; the thermal inactivation of the enzyme at the latter temperature was successfully fitted to a first-order kinetic model, under which a half-life close to 2 h was calculated (Fig. 2b). In comparison, the half-lives of purified PGs from *A. giganteus*, *Trichoderma harzianum*, and *Penicillium viridicatum* at 50°C were reported as 18, 30, and 60 min, respectively [Mohamed et al., 2006; Pedrolli and Carmona, 2010; Silva et al., 2007], while the PG isolated from the thermophilic *Thermoascus aurantiacus* was completely stable upon incubation for 1 h at 50°C, and exhibited a half-life of 10 min at 60°C [Martins et al., 2007]. The *A. sojae* PG is therefore fairly thermostable and suitable for use in fruit juice processing applications. The fact that the clarification process can be generally reduced to 1 h if developed at 50°C [Lea, 1995], and that the purified PG shows a half-life of 2 h at 50°C, makes the enzyme adequate for such purposes. In this context, it is

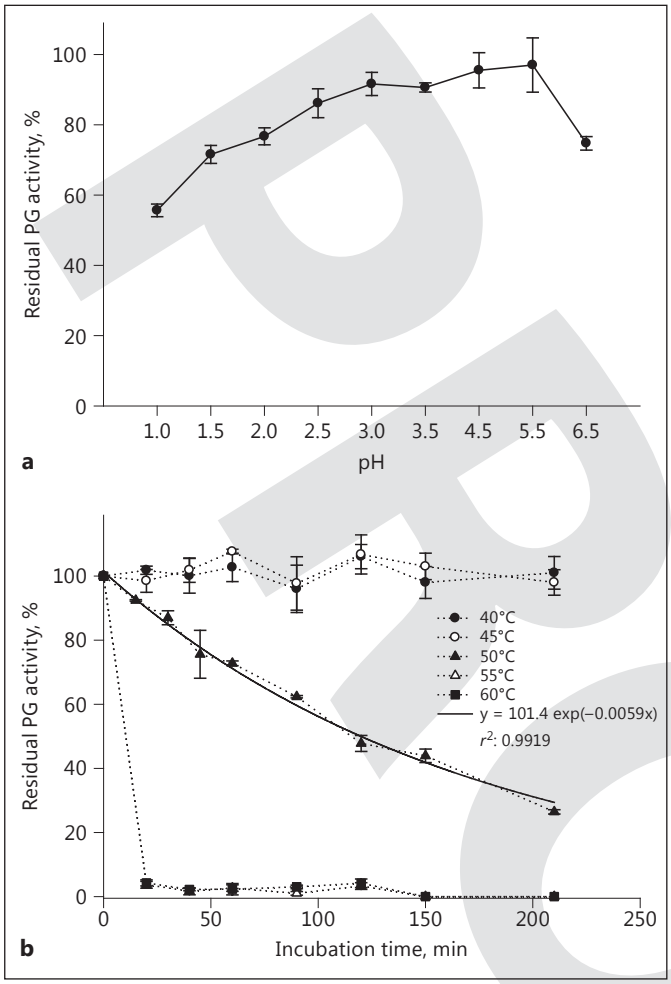

Fig. 2. Influence of pH (**a**) and temperature (**b**) on the stability of the purified *A. sojae* PG. Error bars represent the standard deviation of the mean, calculated for 3 replicates.

also worth mentioning that PG activity of the purified enzyme when assayed at 50°C was 2.3 times higher than measured under standard assay conditions (35°C).

The purified PG acted successfully upon PGA, citrus pectin, and lemon protopectin, being within the pH range for optimum substrate-dependent activity (online suppl. Fig. 2). For PGA, optimum enzyme activity was observed at pH 5.0, while with protopectin as the substrate the enzyme acted best at pH 4.3; as for citrus pectin, optimum activity was found at pH 3.5 but the enzyme still worked at pH 2.3, showing 35% relative activity. Dependence of optimum pH with the substrate assayed was also reported for a purified endo-PG from *A. kawachii* [Contreras Esquivel and Voget, 2004], which at pH 2.0 worked optimally on protopectin while it was almost inactive towards PGA. pH influence studies on enzyme activity with different pectic substances can be useful to predict the proper conditions for different industrial applications; for instance, enzymatic maceration of plant tissues should assure limited pectin degradation and high pectin-releasing (PR) activity, while for fruit juice extraction and clarification, complete destruction of pectin is sought. The relative activity of the purified PG towards PGA decreases considerably at pH 6.5 (online suppl. Fig. 2), whereas a crude PG produced by *A. sojae* with glucose and maltrin as carbon sources still displays 80% relative activity at pH 7.0 [Tari et al., 2008], concluding that these are 2 different enzymes, and indicating that *A. sojae* would produce different PGs depending on the nature of the carbon source used. Differential expression of PG activities was found in *A. kawachii*, which produced and secreted 2 different enzymes whose relative proportion varies markedly if the fungus is grown in a culture medium with glucose or lemon pomace as substrate [Vita et al., 2009].

Regarding metal ions, although some studies have reported their capability to stimulate the activity of fungal/bacterial PGs [Jacob et al., 2008; Pedrolli and Carmona, 2010], when testing Ca^{2+}, K^{+}, Mg^{2+}, and Zn^{2+} we did not find any effect on *A. sojae* PG. In line with the above results, inhibition was not observed in the presence of EDTA, which suggests the enzyme activity apparently does not depend on any free cation. The purified enzyme lost its entire activity when incubated along with Hg^{2+}, which, known for acting as a thiol-blocking agent, indicates a possible involvement of cysteine residues in the substrate binding or in the catalytic processes. On the other hand, PG activity was not affected by β-mercaptoethanol, suggesting that either the tertiary structure of the enzyme does not present disulfide bonds or that these are not accessible to the reducing agent. Besides, neither the incubation with 15% (v/v) ethanol nor with 100 mg/L SO_2 altered the activity of the purified enzyme, which would allow its application during wine making, where SO_2 is normally used to inhibit unwanted yeast and bacteria and to protect wine from excessive oxidation.

Mode of Action

Thin-layer chromatography (TLC) analysis of the hydrolysis products of PGA shows that oligogalacturonates as well as di- and primarily tri-galacturonates were produced during the first stages of the reaction (Fig. 3). After 6 h of incubation, oligogalacturonates were completely degraded, resulting in tri-galacturonate as the major final reaction product, which is consistent with the 34% degradation obtained (online suppl. Fig. 3). These results indicate

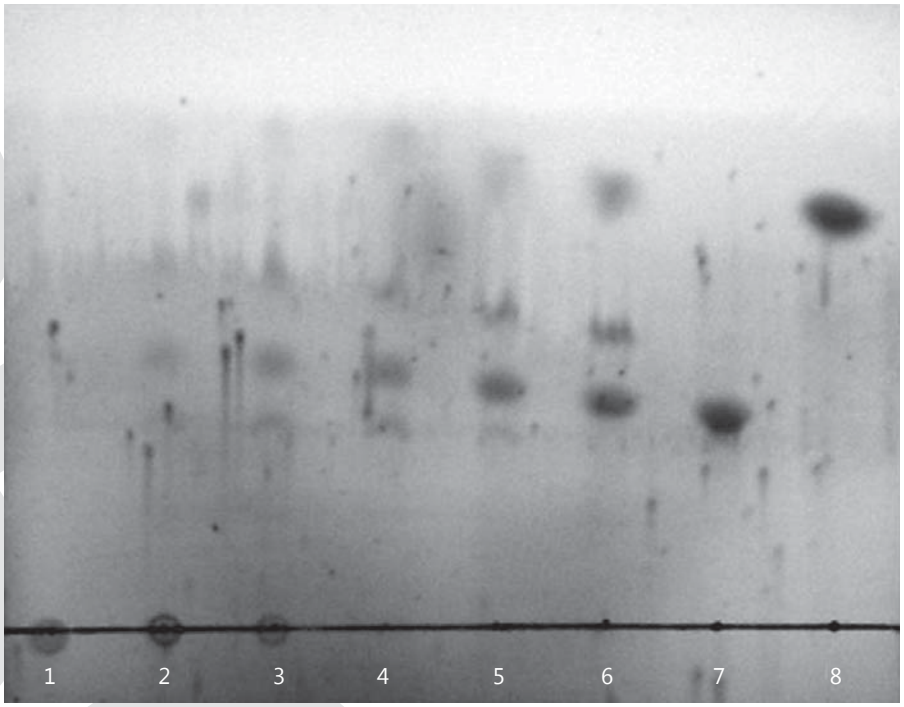

Fig. 3. TLC analysis of the products obtained after 0, 10, 20, 30, 60, and 360 min of hydrolysis of PGA with *A. sojae* PG (lanes 1–6, respectively). Lane 7, tri-galacturonic acid standard; lane 8, GALA standard.

that the enzyme is an endo-PG (EC 3.2.1.15). Although in lesser amounts, mono- and di-galacturonates were also found among the final products, with D-galacturonic acid (GALA) mainly produced at the final stage of the reaction. The substrate degradation pattern exhibited by *A. sojae* endo-PG suggests the enzyme cleaves this substrate in a random fashion but does not display processivity; instead, several random hydrolysis events may occur, ending with the dissociation of the enzyme from its target molecule and the subsequent formation of a new active complex with another polymer chain [Benen et al., 1999]. Furthermore, *A. sojae* endo-PG proves to be incapable of hydrolyzing trimers, which were accumulated at the end of the reaction. The formation of mono- and di-galacturonates would have originated from occasional random hydrolysis events at the first or second glycosidic linkage, the frequency of which increases – especially the cleavage at the first bond – as the size of the transient oligomers decreases.

Kinetic Characteristics

The K_M and V_{MAX} values were 0.134 mg/mL and 9.6 µmol/mg/min, respectively (online suppl. Fig. 4a). This K_M value is among the lowest reported for purified PGs towards PGA [Jayani et al., 2005], indicating a very high affinity for its substrate. A purified PG with slightly higher affinity towards PGA was reported by Niture et al. [2001] from *Fusarium moliniforme* (K_M, 0.12 mg/mL). The turnover constant (k_{cat}) and specificity constant (k_{cat}/K_M) values for this enzyme were also calculated as 7.5/s and 56 mL/mg/s, respectively. Specificity constant values for different reported PGs vary considerably [Dinu et al., 2007; Mohamed et al., 2006; Niture et al., 2001; Semenova et al., 2003], and it has been demonstrated that the use of k_{cat}/K_M for comparing the catalytic effectiveness of enzymes can be misleading [Eisenthal et al., 2007].

We found that *A. sojae* endo-PG was likely to be inhibited by its reaction products because linear progression curves of formation of reducing end groups were hard to obtain when the initial end-product concentration was higher than 350 mg/L. Indeed, the enzyme was found to be competitively inhibited by its end products with an inhibition constant (K_i) of 0.6 mM (online suppl. Fig. 4b), expressed as tri-galacturonate as the major hydrolysis product. The only report of end-product inhibition constants for endo-PGs was reported recently by Mertens and Bowman [2016] as 0.767 mM for the competitive inhibition exerted by di-galacturonate over an endo-PG from *Rhizopus oryzae*. In light of the above results, from a practical point of view a continuous enzymatic process with the enzyme immobilized onto a solid support should be developed when possible, in order to avoid any possible complication related with product accumulation.

Conclusions

An endo-PG from *A. sojae* was successfully purified to a homogenous state as judged by SDS-PAGE and IEF. The enzyme proved to be a robust biocatalyst, as it is stable over a broad pH and temperature range and in the presence of several metal ions. Its maximum activity towards PGA and pectin in acid media, as well as its capacity to solubilize pectin and its tolerance to SO_2 suggests the enzyme can be applied for the extraction and clarification of fruit juices and during winemaking at the prefermentative debourbage stage. Combinations of *A. sojae* endo-PG with other pectinases and carbohydrases may contribute to widen the application range and produce tailor-made enzymatic preparations for specific purposes.

Experimental Procedures

Chemicals

Pectin from citrus fruits, PGA, tri-galacturonic acid, GALA and m-hydroxydiphenyl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Orange peel, supplied by a local citrus company, was dried at 60°C for 2 days, milled to powder with a laboratory mill, and stored at room temperature until use. All other chemicals and reagents used were of analytical grade.

Microorganism and Inoculum

A. sojae ATCC 20235 was obtained from the American Type Culture Collection. The fungus was propagated on YME plates (10 g/L malt extract, 4 g/L yeast extract, 4 g/L glucose, 20 g/L agar), and incubated at 30°C until sporulation. Stock cultures were stored at –80°C. Molasses agar slants were prepared as described in Gögus et al. [2006] and used to obtain the spore suspensions for the seed inoculum. After 1 week of incubation at 30°C, spores were harvested from the slants using 0.02% Tween-80 water and counted in a Neubauer Chamber (Marienfeld, Germany).

Culture Conditions for Enzyme Production

Fermentations were carried out in 1,000-mL Erlenmeyer flasks containing 200 mL of a culture medium composed of ground orange peel (40 g/L) and ammonium sulfate (2.75 g/L) in water. The initial pH of the culture medium was adjusted to 3.3 before inoculation with 1.4×10^5 spores/mL. Cultures were performed at 30°C and 200 rpm in an orbital shaker for 216 h. Samples of 2 mL were withdrawn every 12 h, centrifuged at 6,000 rpm for 20 min and the supernatants assayed for enzyme activities and total protein content determinations. The crude extract used for enzyme purification was obtained as supernatant after withdrawing and centrifuging the entire contents of each flask.

Enzyme Assays and Protein Determination

Enzyme activity measurements were performed at 35°C and pH 5.0, unless otherwise stated. PG activity was determined as described in Cavalitto et al. [1999]. One unit of PG activity was defined as the amount of enzyme that produces 1 μmol of reducing groups as GALA per min.

endoP activity was assayed and calculated according to Gusakov et al. [2002]. One unit of endoP activity was defined as the amount of enzyme required to achieve a 50% reduction in viscosity of the pectin solution in 10 min of reaction.

PL activity was determined spectrophotometrically at 235 nm by measuring the initial rate of formation of unsaturated reaction products from citrus pectin [Edstrom and Phaff, 1964]. One unit of PL activity was defined as the amount of enzyme that releases 1 μmol of 4,5-unsaturated oligogalacturonides per minute.

PE activity was qualitatively determined by adapting the procedure described by Vilariño et al. [1993] to a microplate assay. PR activity was assayed following the procedure outlined in Contreras Esquivel et al. [1999] with slight modifications. Lemon protopectin used as a substrate was prepared as stated in Cavalitto et al. [1997]. The reaction mixture consisted in 20 mg of substrate, 950 μL of 12.5 mM citric acid – 6.25 mM Na_2HPO_4 buffer (CPB), and 50 μL of enzyme sample. After 30 min of incubation in a water bath, the admixture was filtered and the solubilized pectic substances were determined by adding precooled sodium tetraborate to 200 μL of filtrate, boiling the mixture for 10 min and adding 30 μL of m-hydroxydiphenyl solution. The tubes were left at room temperature for 30 min after which absorbance at 525 nm was measured. One unit of PR activity was defined as the amount of enzyme required to solubilize pectic substances equivalent to 1 μmol of GALA per minute.

Citrus pectin and PGA used as substrates were prepared as 5 and 2 g/L solutions, respectively, in 20 mM of acetate buffer (AcB), except for the PE activity assay, in which distilled water was employed. Total proteins were quantified by the Bradford protein method, using bovine-serum albumin as standard.

Enzyme Purification

The crude extract was filtered through cheesecloth and concentrated under reduced pressure at 32°C. The concentrated extract was loaded on a Sephadex G-25 gel-filtration column (General Electric, Little Chalfont, UK) equilibrated with AcB (pH 5.0, 20 mM) and eluted isocratically with the same buffer at a flow rate of 1 mL/min. Collected fractions exhibiting PG activity were pooled and applied to a Q Sepharose FF (General Electric) anion exchange column, preequilibrated with AcB (pH 5.0, 20 mM). Bound proteins were eluted on a linear gradient of NaCl (0.0–1.0 M) in AcB (pH 5.0, 20 mM) over 7 column volumes at a flow rate of 3 mL/min. The collected fractions that showed PG activity were pooled, concentrated by lyophilization and desalted on AcB (pH 3.0, 20 mM) with a column packed with Sephadex G25, prior to its application to an SP Sepharose FF column (General Electric) preequilibrated with the desalting buffer. Elution of the bound proteins was carried out with a 3 mL/min linear gradient of NaCl (0.0–0.5 M) in AcB (pH 3.0, 20 mM) over 5 column volumes. After analyzing the collected fractions, those showing PG activity were pooled, concentrated by lyophilization, resuspended in AcB (pH 5.0, 20 mM) and loaded on a Superdex 75 column (General Electric) preequilibrated with AcB (pH 5.0, 20 mM). Proteins were eluted isocratically at a flow rate of 0.5 mL/min with AcB (pH 5.0, 20 mM), and fractions that resulted positive for PG activity were pooled and stored at –20°C for further characterization. All chromatographic steps were performed with an AKTA-FPLC-U900 system (General Electric).

Electrophoresis

SDS-PAGE was conducted according to Laemmli [1970]. Slab gels were subjected after electrophoresis to either colloidal Coomassie G-250 staining for protein visualization [Neuhoff et al., 1988], or zymogram analysis. For the latter, gels were soaked for 60 min in AcB (pH 5.0, 20 mM) containing 25 g/L Triton X-100, washed with distilled water, and incubated in a 2-g/L PGA solution at 35°C for 45 min. Active bands were visualized as clear zones in a dark background after staining with 0.2 g/L ruthenium red.

Analytical IEF was performed with a Multiphor II IEF unit (General Electric) on a polyacrylamide gel containing 5% acrylamide and 2% ampholytes in a 3–10 pH range. Samples were focused along with pI markers (Broad Range pI Kit, General Electric) for isoelectric point determination. The protein bands were stained with Coomassie R-250.

Effect of pH on Enzyme Stability

The pH stability of the purified enzyme was examined over a range of 1.0–6.5 by measuring the residual PG activity after an incubation step for 2 or 24 h at 20°C in CPB.

Effect of pH on Enzyme Activity

The influence of pH on the activity of the purified enzyme was determined with PGA, citrus pectin, and lemon protopectin within the pH range of 2.3–6.5. Each substrate was prepared or moistened in CPB, preadjusted to the required pH value, and PG or PR activities were measured under the standard assay conditions.

Effect of Temperature on Enzyme Stability

The thermal stability of the purified enzyme was studied by incubation for 210 min at different temperatures, ranging from 40 to 60°C. Aliquots were withdrawn at regular time intervals and the residual PG activity was assayed under standard assay conditions.

Determination of Kinetic Parameters

The kinetic constants K_M and V_{MAX} of the purified enzyme were calculated by fitting the PG activity data determined in a 0.09–9 g/L PGA concentration range to a linear regression after applying the Hanes-Hultin equation [Hultin, 1967]. The inhibition constant, K_i, for the end products of PGA degradation was calculated after applying the Dixon equation [Dixon, 1953] to the PG activity data measured with increasing amounts of the end products, at 0.375 and 0.4375 g/L PGA.

Influence of Metal Ions and Other Chemicals on Enzyme Activity

The effect of Ca^{2+}, Mg^{2+}, Zn^{2+}, K^+, and Hg^{2+} on the enzyme activity was studied by incubating the purified enzyme for 2 h at 20°C in the presence of 1 mM of these metal ions. The effects of β-mercaptoethanol (2 mM), EDTA (5 mM), ethanol (15% v/v), and SO_2 (100 mg/L) were also tested by proceeding as stated above. The PG activity was subsequently measured under standard conditions and expressed as the percent residual activity with respect to a control (100% PG activity).

TLC Analysis

TLC analysis of the enzymatic degradation products was performed on silica gel (60 F254) glass plates (Merck, Kenilworth, NJ, USA), employing n-butanol/acetic acid/water (9:4:7 v/v/v) as the mobile phase. After chromatography, the air-dried plate was submerged into a solution containing 5 g/L of phosphomolybdic acid dissolved in ethanol with 4% sulfuric acid, and heated at 105°C for 5 min. Samples for TLC analysis were obtained by incubating the purified enzyme with 2 g/L of PGA in 20 mM of AcB (pH 5.0), and withdrawing aliquots at several incubation times, which were boiled for 5 min so as to inactivate the enzyme. The reducing end groups were measured and expressed as the amount of GALA, using a GALA standard.

Acknowledgments

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 112-201101-00662) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 2014-1655). D.F. holds a fellowship from CONICET. S.F.C. and I.A.C. are members of the Research Career.

Disclosure Statement

The authors declare that they have no conflicts of interest.

References

Benen JAE, Kester HCM, Visser J: Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II and C. Eur J Biochem 1999;259:577–585.

Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP, Harnett J, Huys G, Laulund S, Ouwehand A, Powell IB, Prajapati JB, Seto Y, Ter Schure E, Van Boven A, Vankerckhoven V, Zgoda A, Tuijtelaars S, Hansen EB: Food fermentations: microorganisms with technological beneficial use. Int J Food Microbiol 2012;154:87–97.

Cavalitto SF, Hours RA, Mignone CF: Quantification of pectin-releasing activity of protopectinase-SE from *Geotrichum klebahnii*. Biotechnol Tech 1997;11:331–334.

Cavalitto SF, Hours RA, Mignone CF: Quantification of protopectinase SE, an endopolygalacturonase with pectin-releasing activity from *Geotrichum klebahnii*. Biotechnol Tech 1999; 13:385–390.

Contreras Esquivel JC, Hours RA, Voget CE, Mignone CF: *Aspergillus kawachii* produces an acidic pectin releasing enzyme activity. J Biosci Bioeng 1999;88:48–52.

Contreras Esquivel JC, Voget CE: Purification and partial characterization of an acidic polygalacturonase from *Aspergillus kawachii*. J Biotechnol 2004;110:21–28.

Dinu D, Nechifor MT, Stoian G, Costache M, Dinischiotu A: Enzymes with new biochemical properties in the pectinolytic complex produced by *Aspergillus niger* MIUG 16. J Biotechnol 2007;131:128–137.

Dixon M: The determination of enzyme inhibitor constants. Biochem J 1953;55:170–171.

Dogan N, Tari C: Characterization of three-phase partitioned exo-polygalacturonase from *Aspergillus sojae* with unique properties. Biochem Eng J 2008;39:43–50.

Edstrom RD, Phaff HJ: Purification and certain properties of pectin trans-eliminase from *Aspergillus fonsecaeus*. J Biol Chem 1964;239: 2403–2408.

Eisenthal R, Danson MJ, Hough DW: Catalytic efficiency and k_{cat}/K_M: a useful comparator? Trends Biotechnol 2007;25:247–249.

Fratebianchi D, Crespo JM, Tari C, Cavalitto S: Control of agitation rate and aeration for enhanced polygalacturonase production in submerged fermentation by *Aspergillus sojae* using agro-industrial wastes. J Chem Technol Biotechnol 2017;92:305–311.

Gainvors A, Nedjaoum N, Gognies S, Muzart M, Nedjma M, Belarbi A: Purification and characterization of acidic endo-polygalacturonase encoded by the *PGL1-1* gene from *Saccharomyces cerevisiae*. FEMS Microbiol Lett 2000; 183:131–135.

Galiotou-Panayotou M, Kapantai M, Kalantzi O: Growth conditions of *Aspergillus* sp. ATHUM-3482 for polygalacturonase production. Appl Microbiol Biotechnol 1997;47: 425–429.

Gögus N, Tari C, Oncü S, Unluturk S, Tokatli F: Relationship between morphology, rheology and polygalacturonase production by *Aspergillus sojae* ATCC 20235 in submerged cultures. Biochem Eng J 2006;32:171–178.

Grassin C, Fauquembergue P: Enzymes, fruit juice processing; in Flickinger MC, Drew SW (eds): Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation. New York, John Wiley & Son, 2009, pp 1030–1061.

Gusakov AV, Markov AV, Grishutin SG, Semenova MV, Kondratyeva EG, Sinitsyn AP: Viscometric method for assaying of total endodepolymerase activity of pectinases. Biochemistry (Moscow) 2002;67:676–682.

Heerd D, Yegin S, Tari C, Fernandez-Lahore M: Pectinase enzyme-complex production by *Aspergillus* spp. in solid-state fermentation: a comparative study. Food Bioprod Process 2012;90:102–110.

Hultin E: Statistical calculation of the accuracy of the Michaelis constant from viscosimetric determinations of polymetaphosphatase and dextranase activity. Acta Chem Scand 1967; 21:1575–1580.

Jacob N, Asha Poorna C, Prema P: Purification and partial characterization of polygalacturonase from *Streptomyces lydicus*. Bioresour Technol 2008;99:6697–6701.

Jayani RS, Saxena S, Gupta R: Microbial pectinolytic enzymes: a review. Process Biochem 2005;40:2931–2944.

Laemmli UK: Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 1970;227:680–685.

Lea AGH: Enzymes in the production of beverages and fruit juices; in Tucker GA, Woods LFJ (eds): Enzymes in Food Processing. Boston, Springer US, 1995, pp 223–249.

Malvessi E, Silveira MM: Influence of medium composition and pH on the production of polygalacturonases by *Aspergillus* oryzae. Braz Arch Biol Technol 2004;47:693–702.

Martins ES, Silva D, Leite RSR, Gomes E: Purification and characterization of polygalacturonase produced by thermophilic *Thermoascus aurantiacus* CBMAI-756 in submerged fermentation. Antonie Van Leeuwenhoek 2007; 91:291–299.

Mertens JA, Bowman MJ: Kinetic properties of *Rhizopus oryzae* RPG1 endo-polygalacturonase hydrolyzing galacturonic acid oligomers. Biocatal Agric Biotechnol 2016;5:11–16.

Mohamed SA, Farid NM, Hossiny EN, Bassuiny RI: Biochemical characterization of an extracellular polygalacturonase from *Trichoderma harzianum*. J Biotechnol 2006;127:54–64.

Neuhoff V, Arold N, Taube D, Ehrhardt W: Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis 1988;9:255–262.

Niture SK, Pant A, Kumar AR: Active site characterization of the single endo-polygalacturonase produced by *Fusarium moniliforme* NCIM 1276. Eur J Biochem 2001;268:832–840.

Ortega LM, Kikot GE, Rojas NL, López LMI, Astoreca AL, Alconada TM: Production, characterization, and identification using proteomic tools of a polygalacturonase from *Fusarium graminearum*. J Basic Microbiol 2014;54: S170–S177.

Pedrolli DB, Carmona EC: Purification and characterization of the exopolygalacturonase produced by *Aspergillus giganteus* in submerged cultures. J Ind Microbiol Biotechnol 2010;37: 567–573.

Semenova MV, Grishutin SG, Gusakov AV, Okunev ON, Sinitsyn AP: Isolation and properties of pectinases from the fungus *Aspergillus japonicus*. Biochemistry (Moscow) 2003; 68:559–569.

Silva D, Martins ES, Leite RSR, Da Silva R, Ferreira V, Gomes E: Purification and characterization of an exo-polygalacturonase produced by *Penicillium viridicatum* RFC3 in solid-state fermentation. Process Biochem 2007;42: 1237–1243.

Tari C, Dogan N, Gogus N: Biochemical and thermal characterization of crude exo-polygalacturonase produced by *Aspergillus sojae*. Food Chem 2008;111:824–829.

van Rensburg P, Pretorius IS: Enzymes in winemaking: harnessing natural catalysts for efficient biotransformations – a review. S Afr J Enol Vitic 2000;21:52–73.

Vilariño C, del Giorgio JF, Hours RA, Cascone O: Spectrophotometric method for fungal pectinesterase activity determination. LWT Food Sci Technol 1993;26:107–110.

Vita CE, Contreras Esquivel JC, Voget CE: Pectolytic enzymes of the industrial fungus *Aspergillus kawachii*. Food Sci Biotechnol 2009;18: 1365–1370.