Purification and Biochemical Characterization of an Extracellular Endopolygalacturonase from *Penicillium frequentans*

Maria de Fátima Borin, Suraia Said, and Maria José V. Fonseca*

Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

An extracellular endopolygalacturonase from extracts of liquid cultures of *Penicillium frequentans* was purified by ion exchange chromatography to homogeneity as judged by electrophoresis and isoelectric focusing. The enzyme is a single subunit glycoprotein with an M_r value of 20 kDa by gel filtration and a p*I* value of 5.6 by electrofocusing. It is active against pectin and sodium polygalacturonate. Comparison of the rate of enzyme-catalyzed increase in fluidity of solutions of substrate vs the rate of release of reducing sugars indicated that the polygalacturonase is endo-acting. The K_m for polygalacturonase is 2.7 g/L, and the V_{max} is 488.28 U of reducing sugar·mg^{-1}. The optimum temperature and pH values under the assay conditions used were 50 °C and pH 4.0−4.7, respectively.

Keywords: *Endopolygalacturonase; Penicillium frequentans*

INTRODUCTION

Pectinases include the depolymerases and pectinesterase (EC 3.1.1.11) which deesterify pectin to pectate and methanol. The depolymerases hydrolyze the glycosidic bonds (hydrolases) or break them by β-elimination (lyases), by random cleavage (endo-acting), or at the extremities (exo-acting) of these polymeric substrates (White and Kennedy, 1988).

These enzymes play an important role in bioconversion as adjuncts to cellulases and/or hemicellulases in the treatment of cellulosic biomass and also in biopulping processes for clarifying fruit juices and liquefying fruits and vegetables (Rombouts and Pilnik, 1986). In addition, the pectinases are used to generate protoplasts in plant biotechnology applications, and these enzymes are involved in the invasion of plant tissues by phytopathogens (Hahn et al., 1989).

Endopolygalacturonase [poly(1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15], a hydrolase which randomly cleaves glycosidic bonds of pectic acids or polygalacturonates, has been purified and characterized from several fungi and bacteria including *Aspergillus niger* (Behere et al., 1993), *Kluyveromyces marxianus* (Barnby et al., 1990), *Penicillium capsulatum* (Gillespie et al., 1990), *Penicillium pinophilum* (Shanley et al., 1993a), *Neurospora crassa* (Polizeli et al., 1991), *Clostridium thermosaccharolyticum* (Rijssel et al., 1993), *Lactobacillus plantarum* strain BA 11 (Sakellaris et al., 1989), and *Botrytis cinerea* (Marcus and Schejter, 1983).

The fungus *Penicillium frequentans* was selected among 100 fungi isolated from soil in different regions of Brazil because of its ability to produce high levels of extracellular pectinases in solid medium using citrus pulp pellets. Pectinase production by this fungus was greater than that of other fungi such as *A. niger*, *Aspergillus nidulans*, and *Rhizopus stolonifer* (Siéssere and Said, 1989). After 17 h of incubation in liquid medium supplemented with citrus pectin, the fungus *P. frequentans* produced higher extracellular pectinase levels than obtained in solid medium, without requiring pregrowth in sucrose or glucose (Said et al., 1991). The extracellular pectinases produced were separated by ion exchange chromatography into three exopolygalacturonases, three endopolygalacturonases, and pectinesterase (Siéssere et al., 1992) and resolved by electrophoresis (Fonseca and Said, 1995).

This paper describes the purification and characterization of an extracellular endopolygalacturonase (endo-PGI) produced by *P. frequentans*. The structural and catalytic properties of the enzyme are discussed.

MATERIALS AND METHODS

Microorganism and Cultivation Conditions. The organism used in these studies, *P. frequentans*, was isolated from Brazilian soil samples and deposited in the collection of the Fundação Tropical de Pesquisa e Tecnologia "André Tosello" (Campinas, SP, Brazil) under the number FTPT 2037. It was routinely subcultured on oats-agar. The procedure for the preparation of inoculation and liquid culture was as described by Siéssere et al. (1992).

Separation and Purification of the Endopolygalacturonase Enzyme. The mycelium was separated from the culture fluid by filtration. The culture filtrate (133 mL) retained in dialysis bags was concentrated (about 3.8-fold) by overlaying dialysis bags with crystalline sucrose at 4 °C (Cleveland and McCormick, 1987). The concentrate was dialyzed against 25 mM Tris/acetate buffer, pH 6.5, containing 1 mM EDTA (buffer A).

The crude extracellular enzyme (viscosimetric activity, 122.75 UV; protein, 5.7 mg) was subjected to chromatography on DEAE-Sephacel (1.8 × 61.0 cm) preequilibrated with buffer A. Proteins not bound to the resin were eluted with the same buffer; bound proteins were eluted by stepwise increases in NaCl concentration in buffer A (20, 150, and 300 mM) at a flow rate of 42.6 mL·h^{-1}, and 5 mL fractions were collected. The fractions of unbound proteins, showing viscosimetric and reducing end group-releasing activities, were pooled and dialyzed against 25 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA (buffer B).

The dialyzed enzyme was applied to a column of CM-Sepharose (1.8 × 37.0 cm) preequilibrated with buffer B. After unbound proteins were eluted with the same buffer, the bound proteins were eluted by stepwise increases in NaCl concentration in buffer B (20, 50, and 75 mM) at a flow rate of 100

* Author to whom correspondence should be addressed at Av. do Café s/n, 14040-903 Ribeirão Preto, São Paulo, Brazil [telephone (016) 633 3036, ext. 389; fax (016) 633 1092; e-mail magika@usp.br].

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mL·h^{-1}, and 5 mL fractions were collected. Fractions containing viscosimetric and reducing end group-releasing activities were pooled, dialyzed against buffer B, and rechromatographed on a CM-Sepharose column as described above.

The purified preparations (endo-PGI) obtained were used for homogeneity checks and determination of physical and chemical properties.

Enzyme Assay. *Viscosimetric Activity.* This was determined at 30 °C in an Ostwald viscosimeter with 0.25% pectin solution, according to Siéssere et al. (1992). One viscosimetric unit (VU) is expressed as the amount of enzyme that reduced the initial viscosity of the pectin solution by 50% in 1 min.

Reducing Releasing Activity. This was assayed with sodium polypectate as a substrate by measuring the amount of reducing sugar released (Miller, 1959) as previously described (Siéssere et al., 1992). An enzyme unit is the amount that releases reducing sugar at an initial rate of 1 μmol·min^{-1} at 37 °C, using monogalacturonic acid as the standard.

Electrophoresis. *Native PAGE and Pectic Enzyme Identification.* Electrophoresis of culture filtrate and fraction chromatographies, using a vertical gel electrophoresis unit (Sigma E-4266), were carried out on a 12% (w/v) polyacrylamide slab gel (8 × 13 cm) containing 0.1% (w/v) pectin or 0.2% (w/v) sodium polypectate (NaPP) and a 4% stacking gel using the discontinuous buffer system of Davis (1964). The electrophoresed gel was incubated in a 0.1 M malic acid solution at 50 °C for 18 h. Pectic enzymes were stained by the ruthenium red staining procedure according to Cruickshank and Wade (l980).

SDS−PAGE. The purified enzymatic preparation was examined for homogeneity and M_r value by electrophoresis under denaturing conditions in 4% and 14% (w/v) polyacrylamide slab gels as described by Laemmli (1970) using running Tris-glycine buffer, pH 8.3, with 0.1% SDS. Proteins in the gels were stained with Coomassie brilliant blue R250. Molecular weights were calculated as described by Weber and Osborn (l969) with reference to the following standard protein markers: lactalbumin, 14.2 kDa; trypsin inhibitor, 20.1 kDa; trypsinogen, 24 kDa; carbonic anhydrase, 29 kDa.

Isoelectric Focusing. For determination of the p*I* value and homogeneity of the purified enzymatic preparation, slab gel isoelectric focusing was performed in 5% polyacrylamide gel, with thickness of 1 mm, containing 0.12% ampholine (p*I* 3.0−10.0) and 0.28% ampholine (p*I* 4.0−6.0). The anodic electrolyte was 20 mM glutamic acid and the cathodic electrolyte 2 M ethanolamine (procedures recommended in the Bio-Rad pratical information sheet). Samples containing 0.2−28.0 μg of protein were applied to slab gel. Electrofocusing was performed at a constant voltage of 800 V until the current flow decreased below 1.5 mA at 5 °C. The electrofocused gel was cut into two parts. One part was incubated with a pectin-agarose overlay for 3 h at 50 °C, and then the overlay was immersed in 0.05% (w/v) ruthenium red solution for detection of pectinolytic activities (Ried and Collmer, 1985). For determination of p*I*-purified enzyme, the other part of the electrofocused gel, containing 28 μg of protein, was sliced into 5 mm sections, and each was triturated and suspended in 1.0 mL of water. The viscosimetric activity determinations and pH were determined.

Gel Filtration. The molecular mass of the purified endo-PGI was estimated by filtration through BioGel P60 (1.8 × 60.0 cm) equilibrated with buffer B. Elution (6.0 mL·h^{-1}) was carried out with the same buffer, and fractions (1.5 mL) were collected and analyzed for viscosimetric and reducing end group-releasing activities. Bovine serum albumin, 67 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa, and cytochrome *c*, 12.4 kDa, were included as molecular mass standards.

Effects of pH and Temperature on Activity of Purified Endo-PGI. The effect of pH on the viscosimetric activity of endo-PGI was determined by adding 0.05 mL of purified enzyme solution (0.1 U of viscosimetric activity; 2.5 μg of protein) to 5.5 mL of 0.2% (w/v) pectin in the following buffers: 0.05 M HCl/KCl (pH 1.0−2.2), 0.05 M glycine/KCl (pH 2.6−3.6), 0.05 M sodium acetate (pH 3.6−5.6), 0.05 M sodium phosphate (pH 5.8−8.0), 0.1 M Tris/HCl (pH 7.2−9.0), and 0.05 M carbonate/bicarbonate (pH 9.2−10.6). The mixture was incubated at 50 °C for 10 min, and viscosimetric activity was assayed.

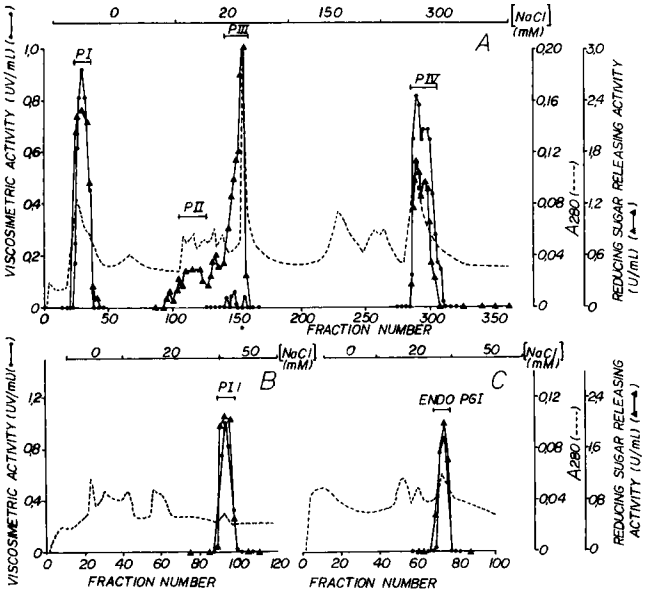

Figure 1. Separation profiles on DEAE-Sephacel (A) and CM-Sepharose (B and C) of pectinolytic activity produced by *P. frequentans*. (A) The concentrated and dialyzed culture fluid was applied to a DEAE-Sephacel column (1.8 × 61.0 cm) preequilibrated with 25 mM Tris/acetate buffer, pH 6.5, containing 1 mM EDTA. Fractions (5.0 mL) were collected and assayed for A_{280} (- - -), viscosimetric (●), and reducing sugar-releasing (▲) activities. (B) Fractions 20−40 showing enzymatic activity were pooled (PI) and applied to a CM-Sepharose column (1.8 × 37.0 cm) preequilibrated with 25 mM acetate buffer, pH 5.0, containing 1 mM EDTA. Fractions (5 mL) were collected and assayed for A_{280} (- - -), viscosimetric (●), and reducing sugar-releasing (▲) activities. (C) Fractions 70−80 containing viscosimetric and reducing sugar-releasing activities were pooled (PI.1) and rechromatographed on a CM-Sepharose column as described above.

For the determination of the effect of temperature on viscosimetric activity, 0.05 mL of endo-PGI (0.1 U of viscosimetric activity; 2.5 μg of protein) was added to 5.5 mL of 0.2% pectin in buffer B, and this mixture was incubated at 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C for 10 min. The viscosimetric activity was then assayed.

Kinetic Study. The Michaelis constants (K_m) of the purified enzymes were determined with various concentrations of substrate (sodium polygalacturonate), and the data obtained were plotted according to the Lineweaver−Burk plot.

Analytical Methods. Protein quantitation was performed by the method of Bensadoun and Weinstein (1976), with bovine serum albumin as standard. In chromatographic steps, protein was followed by its absorbance at 280 nm. Total carbohydrate in protein samples was estimated by the phenol/sulfuric acid method of Dubois et al. (1956) using glucose as standard.

RESULTS AND DISCUSSION

Purification of Endo-PGI. A simple and rapid three-step procedure was adopted to separate and purify the endo-PGI of *P. frequentans* (Figure 1). The purification of this enzyme from 133 mL of culture filtrate is summarized in Table 1. The final preparation, which was free from pectinesterase activity and apparently homogeneous by SDS−PAGE and isoelectric focusing, represented a 4.0-fold purification and a yield of about 20.5% with respect to the starting material. The presence of other polygalacturonases that also degraded pectin (Fonseca and Said, 1995) and the low level of protein detected in the culture filtrate resulted in low

Table 1. Purification of Extracellular Polygalacturonase Activity

step	volume (mL)	total protein (mg)	total activity (UV)	specific activity [UV·(mg of protein)$^{-1}$]	purification factor (fold)	yield (%)
crude filtrate	133.00	12.59	174.67	13.87	1.00	100.00
pooled DEAE-Sephacel (PI)	90.86	1.63	69.46	42.61	3.07	39.77
pooled CM-Sepharose (PI.1)	58.82	0.83	29.47	35.51	2.56	16.87
pooled CM-Sepharose (PGI)	30.04	0.67	35.98	58.04	4.18	20.59

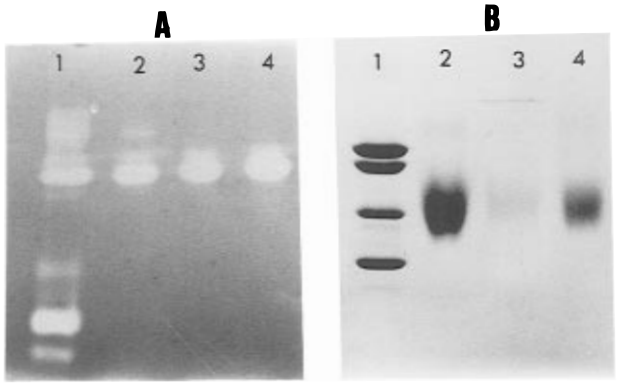

Figure 2. Electrophoretic analysis of the endo-PGI by PAGE (A) and SDS−PAGE (B). (A) Lane 1 (culture fluid, 30 μg of protein), lane 2 (PI, 10 μg of protein), lane 3 (PI.1, 10 μg of protein), and lane 4 (endo-PGI, 10 μg of protein). (B) Protein samples: lane 1, molecular weight markers; lane 2, endo-PGI (3.94 μg of protein); lane 3, endo-PGI (0.50 μg of protein); and lane 4, endo-PGI (1.57 μg of protein).

purification, despite the homogeneity of endo-PGI. These results suggest that probably almost all proteins secreted by *P. frequentans* at the established growth conditions were pectic enzymes. The purified endo-PGI showed, as characteristics, not to bind to the anion exchange resin and to be the most abundant of the pectinases produced by *P. frequentans*. Other polygalacturonases such as the PG from *Phanerochaete chrysosporium* (Shanley et al., 1993b), PGIII from *P. pinophilum* (Shanley et al., 1993a), and PG from *N. crassa* (Polizeli et al., 1991) showed similar characteristics.

Homogeneity, M_r, and p*I* Values. By SDS−PAGE, endo-PGI exhibited one broad band of M_r 19 ± 3 kDa (Figure 2). By gel filtration, compared with the behavior of standard proteins, the M_r value of endo-PGI was calculated to be 20 ± 2 kDa (BioGel P60). The M_r of endo-PGI was lower than those of fungal endopolygalacturonases that were in the range of 30−85 kDa (Rombouts and Pilnik, l980; Whitaker, 1990).

Analytical nondenaturing PAGE of the purified preparation revealed two activity bands, one more deeply stained than the other. This preparation reacted positively to the colorimetric method for determination of carbohydrate indicating a glycoprotein nature. The carbohydrate content was estimated at 20%.

The presence of the two activity bands might be due to the microheterogeneity of the carbohydrate moiety present in endo-PGI that may affect the physicochemical properties of proteins by changing their charge, mass, size, and hydrophobicity. This microheterogeneity was a common finding with purified fungal extracellular enzymes (Coughlan, 1985). The results suggest that endo-PGI was a single-unit glycoprotein with a molecular mass of about 20 kDa.

Endo-PGI showed a single activity band, for p*I* value 5.6, upon isoelectric focusing (Figure 3). This value was within the range of p*I* values obtained for endopolygalacturonases from different fungi.

pH and Temperature Optima and Kinetic Properties. The optimum pH of the endo-PGI was 4.0−4.7, and at pHs above 6.0 the *P. frequentans* enzyme was inactive. The values reported in the literature for optimum pH for fungal endopolygalacturonases are pH 3.8−6.5, and in general, activity drops off more rapidly on the alkaline side than on the acid side of this value (Rombouts and Pilnik, 1980).

The optimum temperature value for the purified enzyme under the standard assay conditions (pH 5.0 and 10 min incubation time) was determined to be 50 °C. The determination of the activation energy for the hydrolysis of pectin (determined by viscosimetric activity) showed a typical Arrhenius plot, and the E_a found was 6.42 kcal/mol (26.89 kJ/mol). A sudden drop indicated protein denaturation. The same effect was observed in the Arrhenius plot when E_a was determined for hydrolysis of sodium polypectate (determined by the reducing sugar-releasing activity). However, this plot showed a sharp change in the slope at 37 °C (transition temperature). The E_a, observed for temperatures below transition temperature, was 15.496 kcal/mol (64.87 kJ/mol), and for temperatures above that, it was 2.35 kcal/

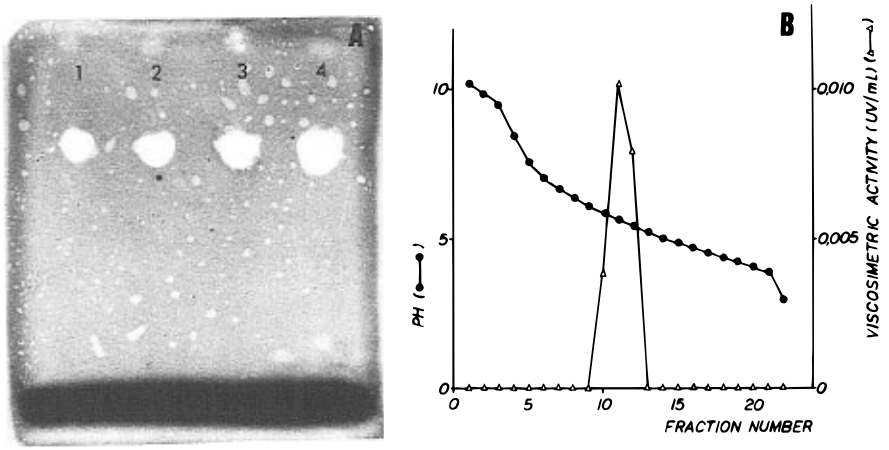

Figure 3. (A) Electrofocusing of the endo-PGI in slab polyacrylamide gel: lanes 1, 0.2 μg of protein; 2, 0.3 μg of protein; 3, 0.5 μg of protein; and 4, 0.7 μg of protein. (B) Determination of p*I*-purified enzyme: viscosimetric activity (△) and pH (●).

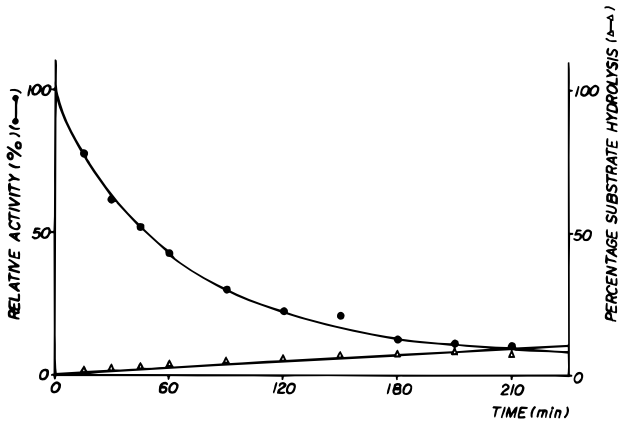

Figure 4. Viscosity change and sodium polypectate hydrolysis by the purified endo-PGI as a function of time: (●) relative viscosity (%) and (△) hydrolysis of glycosidic bonds (% of nominal monomer concentration).

mol (9.82 kJ/mol). The purified enzyme showed good stability at −20 °C, losing <10% activity after 18 months.

The rate of the enzyme-catalyzed reduction in viscosity of solutions of pectin or sodium polypectate was considerably greater than the rate of release of reducing sugars from these substrates (Figure 4). This property is a characteristic of random-acting, i.e., endo-acting, enzymes, and this result is similar to that obtained by Siéssere et al. (1992), using nonpurified preparations of the enzyme. Moreover, as judged by the decrease in viscosity, the ability of the endo-PGI to hydrolyze NaPP was similar to that of pectin. Thus, the *P. frequentans* enzyme was different from other fungal polygalacturonases, such as the endopolygalacturonases of *N. crassa* (Polizeli et al., 1991), *P. capsulatum* (Gillespie et al., 1990), *P. chrysosporium* (Shanley et al., 1993b), and *P. pinophin* (Shanley et al., 1993a) that were more active against NaPP than pectin.

The kinetic properties of the endo-PGI were determined at 50 °C using NaPP concentrations ranging from 5.0 to 10.0 $g \cdot L^{-1}$. Lineweaver−Burk plots gave an apparent K_m of 2.7 $g \cdot L^{-1}$ and an apparent V_{max} of 488.28 $U \cdot mg$ of $protein^{-1}$. The K_m of the *P. frequentans* enzyme was lower than that of polygalacturonases produced by *N. crassa* (Polizeli et al., 1991), *P. capsulatum* (Gillespie et al., 1990), and PGI and PGII of *P. pinophilum* (Shanley et al., 1993b), but it was higher than that of PGIII produced by *P. pinophilum*.

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