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Purification and Partial Characterization of an Exo-polygalacturonase from *Paecilomyces variotii* Liquid Cultures

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Abstract An extracellular polygalacturonase (PG) produced from *Paecilomyces variotii* was purified to homogeneity through two chromatography steps using DEAE-Fractogel and Sephadex G-100. The molecular weight of *P. variotii* PG was 77,300 Da by gel filtration and SDS-PAGE. PG had isoelectric point of 4.37 and optimum pH4.0. PG was very stable from pH3.0 to 6.0. The extent of hydrolysis of different pectins by the purified enzyme was decreased with an increase in the degree of esterification. PG had no activity toward non-pectic polysaccharides. The apparent K_m and V_{max} values for hydrolyzing sodium polypectate were 1.84 mg/mL and 432 μmol/min/mg, respectively. PG was found to have temperature optimum at 65 °C and was totally stable at 45 °C for 90 min. Half-life at 55 °C was 50.6 min. Almost all the examined metal cations showed partial inhibitory effects under enzymatic activity, except for Na^{+1}, K^{+1}, and Co^{+2} (1 mM) and Cu^{+2} (1 and 10 mM).

Keywords *Paecilomyces variotii* · Exoenzymes · Pectinases · Polygalacturonase · Pectin

Introduction

Celluloses, hemicelluloses, and pectic substances are the most abundant carbohydrates of plant cell walls. Pectic substances like pectin, protopectin, and pectic acids are present in the middle lamella and contribute to firmness and structure of plant issues. In pectic substances, D-galacturonic acid units are linked by α-1,4-glycosidic linkages, and the extent of esterification carbonyl substituents ranges from 60% to 90% [1].

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Pectins are naturally hydrolyzed by pectinolytic enzymes, which have been classified on the basis of their galacturonan backbone attack mechanism. Pectin-degrading enzymes can be divided into esterases and depolymerases [2]. The first group is represented by pectin esterase (PE) and the second by polygalacturonase (PG) and pectin lyase (PL). Polygalacturonase (EC 3.2.1.15) catalyzes the hydrolysis of α-(1,4) glycosidic bounds between non-esterified galacturonic acid units [3]. Pectate lyase (EC 4.2.2.2) and pectin lyase (EC 4.2.2.10) cleave α-1,4-glycosidic linkages by trans-elimination, which results in galacturonide with an unsaturated bond between C4 and C5 at the non-reducing end of the galacturonic acid formed.

Pectinases can be used in several industrial processes such as clarification of fruit juices, olive oil recovery, wine production, vegetable oil extraction, functional foods, improvement of cassava starch extraction, tea and coffee fermentation, textile industry, paper and cellulose industry (in addition to cellulases [4]), and animal feeds [1].

Although pectinases are used in industrial processes in crude form, their purification and the knowledge of the biochemical characteristics of these enzymes are essential for the understanding of their structure, mechanisms of action, and thermostability. The aim of this work was to study a PG from the fungus *Paecilomyces variotii*, which was recently isolated from decompositing materials. This fungus presents a thermotolerant character with excellent development at high temperatures. Besides the purification of a novel *P. variotii* thermostable PG, this study reports some physical–chemical characteristics that affect the stability, enzymatic activity, and kinetic properties, which stimulate the approval of this enzyme in biotechnological applications.

Materials and Methods

Microorganism

P. variotii strain used in this study was isolated from decaying hemicellulosic material collected in the state of São Paulo, Brazil, and was maintained on solid oatmeal slants as a stock culture, at 4 °C. This fungus was classified and deposited as *P. variotii* at the culture collection of the Recife Mycology University—PE, Brazil, WFCC, number 604.

Growth Conditions and Enzyme Production

For inoculum preparation, the surfaces of oatmeal solid media contained in Petri plates were scraped with platinum inoculating loops in the presence of distilled water. The spore solution obtained was counted in a cell-counting chamber, and a concentration of 3×10^5 spores/mL was used as inoculum into 125-mL Erlenmeyer flasks containing 25 mL of liquid Czapeck medium [5] with 1.0% citric pectin (*w*/*v*) or other carbon sources. The cultures were incubated at 40 °C, under orbital shaking (100 rpm), for 5 days, when PG activity reached the maximum. Besides this medium, other growth media were Khanna [6], SR [7], Adams [8], and Vogel [9]. Culture filtrates were obtained from filtration through Whatman No. 1 paper in a Buchner funnel. The filtrate was used as a source of crude extracellular PG.

PG Purification

Approximately 140 mL of crude enzyme solution was dialyzed against 10 mM Tris–HCl buffer, pH 7.5, overnight. The material was loaded on a DEAE-Fractogel column (1 × 5 cm) equilibrated with 10 mM Tris–HCl buffer, pH 7.5. The unabsorbed material was removed

by washing the column with 50 mL of the same buffer. The enzyme was eluted with a linear gradient from 0.0 to 0.5 M NaCl (150 mL) in the same buffer at a flow rate of 30 mL/h. Fractions of 10 mL were collected. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. One peak of active fractions was pooled and designated PG. This pool was dialyzed against 50 mM sodium acetate buffer, pH5.0, concentrated by lyophilization. The material was suspended in 2 mL of 50 mM sodium acetate buffer, pH4.0 and loaded on Sephadex G-100 column (2×85 cm) equilibrated with the same buffer, and 1 mL fractions were collected at a flow rate of 12 mL/h.

Enzymatic Assays and Protein Determination

PG activity was assayed with a 1% solution of sodium polypectate (NaPP) in 100 mM sodium acetate buffer (pH4.0) at 65°C for 10 min. The amount of reducing groups, expressed as galacturonic acid released by enzymatic action, was quantified by the DNS method [10]. One unit of enzyme activity (U) was defined as the amount of enzyme releasing 1 μmol of galacturonic acid/min. The blank contained heat-inactivated crude enzyme. Protein concentration was determined according to Lowry et al. [11], using bovine serum albumin as standard. The values were expressed as total protein, where milligrams per milliliter was multiplied by the sample total volume.

Analytical Electrophoresis

Electrophoresis under non-denaturing conditions was performed in 12% (*w*/*v*) acrylamide slab gel according to the method of Davis [12] using a Tris–glycine buffer, pH8.3. Protein bands were located by staining with silver nitrate.

Molecular weight was determined by gel filtration using HPLC with Bio-sil SEC-400 column (30×0.78 cm), equilibrated and eluted with 100 mM HEPES buffer, pH6.8, containing 150 mM NaCl and 10 mM sodium azide, at a flow rate of 1.0 mL/min. Fractions of 1 mL were collected. The column was calibrated using some standard proteins: globulin (158 kDa), ovoalbumin (45 kDa), and myoglobin (17 kDa). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis [13]. Electrophoresis was carried out on a 12% polyacrylamide gel using Tris–glycine, pH8.3. Protein bands were located by staining with silver nitrate. SDS-denatured molecular weight markers were used for the calibration curve.

Isoelectric Focusing

The isoelectric point (p*I*) of the enzyme was determined by isoelectric focusing on 5% polyacrylamide gel as described by O'Farrel et al. [14]. Pharmalyte® carrier ampholytes (pH2.0 to 5.0) were used for the calibration curve. After electrophoresis at 500 V, 6 h, at room temperature, the gels of the control and sample were cut in fragments of 0.5 cm, incubated in KCl 25 mM solution overnight and the pH was measured. The fragments of the gel with the enzyme were macerated in the presence of buffer assay, and the enzymatic activity was measured.

Enzyme Characterization

PG activity was assayed as a function of pH, in McIlvaine buffer (pH2.5–8.0), at 60°C with 1% NaPP as substrate. The effect of temperature on PG activity was determined in 100 mM

sodium acetate buffer, incubated at temperatures between 30 and 80 °C, at the pH optimum (4.0). The thermal stability was investigated by measuring the residual activity of the enzyme after 90 min of incubation at 45, 50, and 55 °C. The half-life was determined by incubating the enzyme at 55 °C for 90 min at the pH and temperature optima.

pH stability of the purified enzyme was evaluated by dispersing (1:1) enzyme solution in McIlvaine buffer solutions pH2.5–8.0 and by maintaining these solutions at 25 °C for 24 h. An aliquot was taken to determine the remaining activity at the optimum pH and temperature.

To determine substrate hydrolysis, solutions of NaPP and citric pectin with 9% to 70% degree of esterification (DE) were used as substrates under optimal conditions for enzyme activity.

The Michaelis constant K_m and V_{max} values were determined from Hanes [15] plots of enzyme activity measured with NaPP as substrate, at concentrations between 0.2 and 12 mg/mL.

The effect of various metal cations on enzyme activity was evaluated at a concentration of 1 and 10 mM in the reaction mixture, with $HgCl_2$, $ZnCl_2$, $MnCl_2.4H_2O$, $Fe_2SO_4.4H_2O$, NaCl, $MgCl_2.6H_2O$, $AlCl_3$, $AgNO_3$, $Pb(C_2H_3O_2).3H_2O$, KCl, $CuCl_2$, and $CoCl_2.6H_2O$.

The products of the hydrolysis of NaPP by PG were analyzed by thin layer chromatography (silica gel/TLC-cards with 254 nm fluorescence indicator; 0.2 mm layer thickness; 20×20 cm aluminum cards-Fluka), with *n*-butanol/acetic acid/water (5:3:2 *v*/*v*) as the mobile phase. Detection was accomplished by spraying the dried plate with 0.2% orcinol (*w*/*v*) dissolved in methanol and sulfuric acid at 9:1, followed by heating at 100 °C for 5 min.

Reproducibility of the Results

All data are the mean of at least three independent experiments.

Results and Discussion

Purification of PG

PG was purified to apparent homogeneity from the culture filtrate with only two purification steps: ion-exchange chromatography and gel filtration. Only one activity peak was found in the eluted fractions from DEAE-Fractogel column (Fig. 1). The elution was carried out with 217 mM NaCl. This step resulted in 2.52-fold enzyme purification and 51% yield and removed the pigments from the original culture filtrate. In the second step, the PG was separated by Sephadex G-100 column. Only one peak was observed (Fig. 2) and resulted in 10.1-fold purification of the enzyme, giving a specific activity of 298.5 (U/mg protein) and final yield of 47.2%. A summary of the purification procedure is given in Table 1.

The presence of only one PG isoform in the culture filtrates seems to be a peculiarity of *P. variotii* since multiple isoforms of secreted enzymes are normally obtained in fungal culture media [16]. Mohamed et al. [3] described a PGII purification from *Trichoderma harzianum* after culture filtrate elution on DEAE-Sepharose and Sephacryl S-200 and resulted in 13-fold purification, giving a specific activity of 276 (U/mg protein) and final yield of 55%. On the other hand, a PGII from *Penicillium frequentans* was purified in DEAE-Sephacel column and resulted in a 4.95-fold purification with a specific activity of 249.4 U/mg protein.

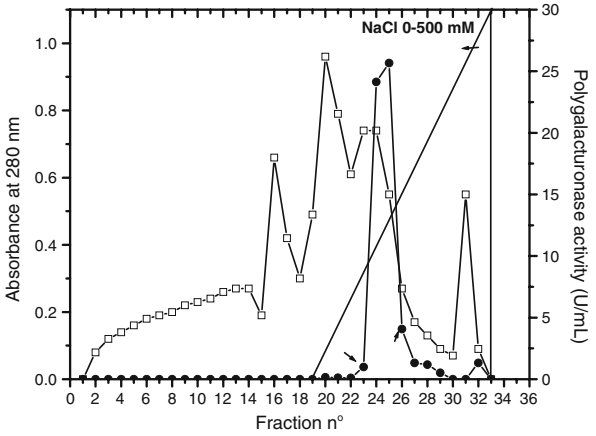

Fig. 1 A typical elution profile for the chromatography of *P. variotii* PG on DEAE-Fractogel column previously equilibrated with 10 mM Tris–HCl buffer, pH7.5. *Squares* Absorbance at 280 nm; *circles* PG activity

Homogeneity, Molecular Weight, and p*I*

The homogeneity of the purified PG was demonstrated by the presence of one single protein band on polyacrylamide gel (Fig. 3a). The molecular weight of *P. variotii* PG was estimated to be 77,300 Da by gel filtration. This molecular weight was confirmed by SDS-PAGE (Fig. 3b) and estimated to be 77,300 Da as a single subunit. This observation is similar to the result reported for PG from *Fusarium oxysporum* f.sp. *lycopersici* [17]. In comparison with some *Trichoderma* sp., lower molecular weights were detected for *Trichoderma reesei* PG1 (66,000 Da) and PG2 (63,000 Da) [18]. The highest molecular weights were detected for PG from *Bacillus* sp. (115,000 Da) [19]. Generally, microbial

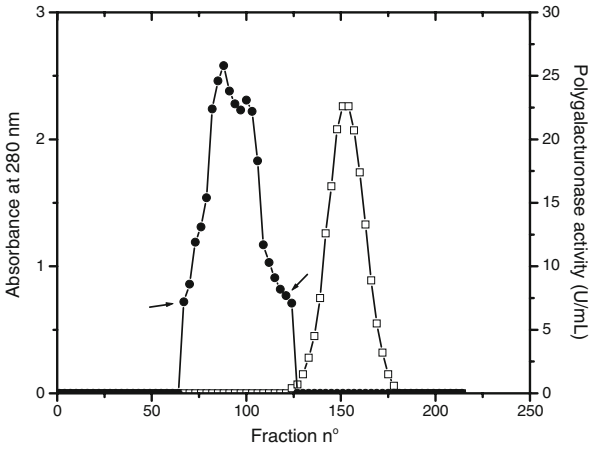

Fig. 2 A typical elution profile for the chromatography of *P. variotii* PG on Sephadex G-100 column (2× 85 cm) previously equilibrated with 50 mM sodium acetate buffer, pH4.0. *Squares* Absorbance at 280 nm; *circles* PG activity

Table 1 Purification of the PG produced by *P. variotii*.

Steps	Vol (mL)	Proteins[a] (total mg)	Activity (total units)	U/mg protein	Recovery (%)	Purification factor
Culture filtrate	140	48.9	1,453.2	29.7	100	1
DEAE-Fractogel	40	9.9	741.4	74.8	51.0	2.5
Sephadex G-100	40	2.3	686.5	298.5	47.2	10.1

[a] Proteins were estimated by Lowry method, where milligrams per milliliter was multiplied by the culture filtrate total volume (total mg)

PGs have acidic p*I* [19, 20] or alkaline p*I* [21, 22]. Similarly, the isoelectric focusing experiment indicated that *P. variotii* PG migrated as a single major band with acidic isoelectric point (p*I*) of about 4.37 (Fig. 3c).

Characterization of the Purified *P. variotii* PG

pH Optimum

The effect of pH on the *P. variotii* PG activity toward PGA was examined at 60 °C. As shown in Fig. 4a, the enzyme showed hydrolase activity from pH2.5 to 8.0. The optimum pH for the activity was 4.0, and the enzyme retained above 80% of maximum activity from pH2.5 to 5.0. The same pH optimum was reported for PG from *T. reesei* PG1 [18].

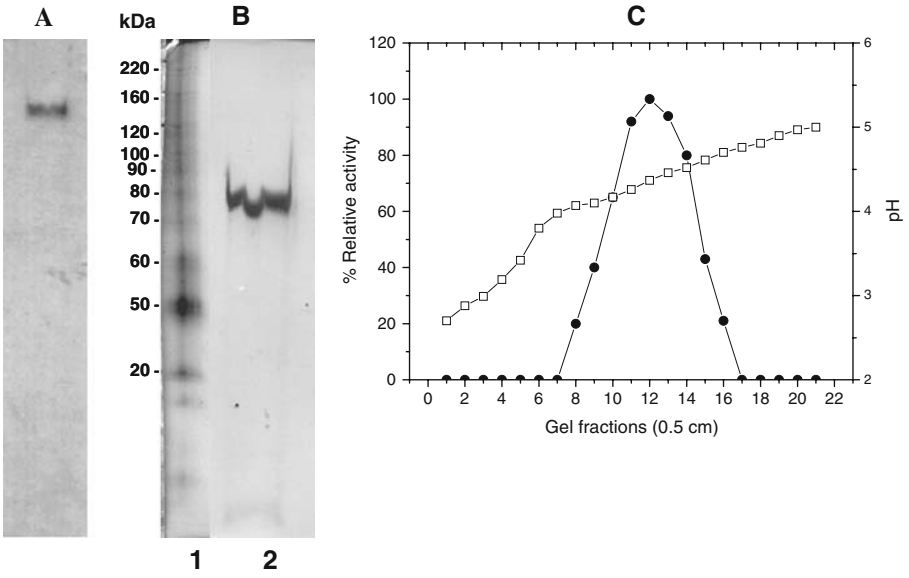

Fig. 3 Electrophoretic patterns for *P. variotii* PG. **a** Non-denaturing 12% PAGE; **b** 8% SDS-PAGE for molecular weight, standard (*1*) and purified PG after elution in Sephadex G-100 (*2*); **c** isoelectric point (*squares*) gel fractions pH and (*circles*) PG activity

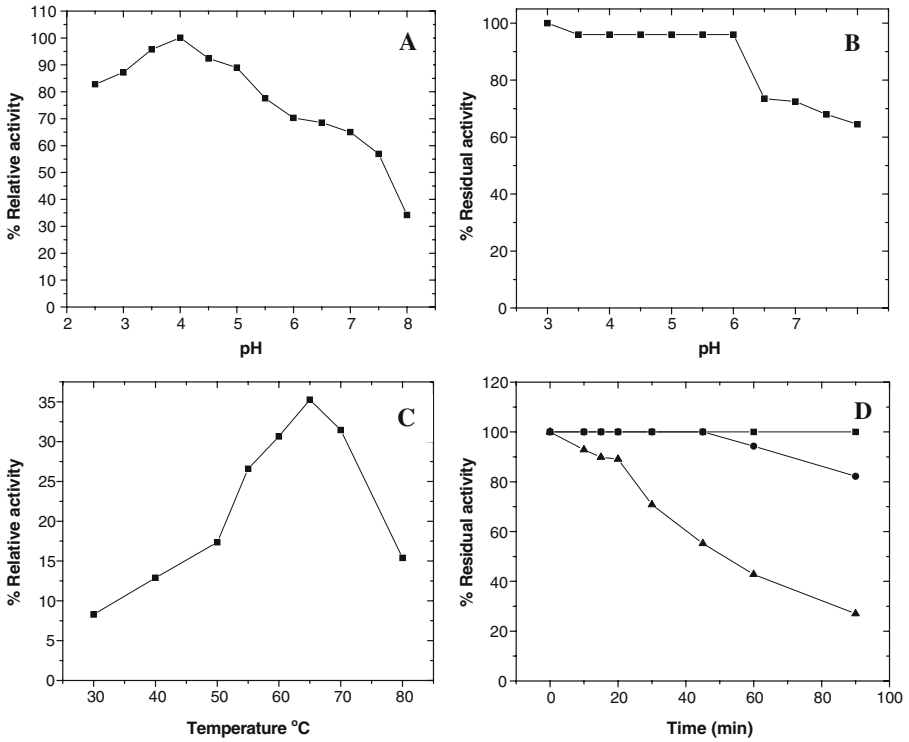

Fig. 4 Effect of pH on activity (**a**) and stability (**b**) of PG; effect of temperature on activity (**c**) and stability (**d**) of PG. *Squares* 45°C, *circles* 50°C, *triangles* 55°C

According to most of the reports, a lot of endo- and exo-PGs forms had the same pH optimum range (4.0–6.5) [2]. Sakamoto et al. [23] reported that the optimum activities occurred at pH3.4–3.8 for exo-PG1 and pH3.4–4.2 for exo-PG2 from *Aspergillus niger*, respectively [23]. In contrast, the PG from *Aspergillus kawachii* had an optimum activity at a low pH (2.0–3.0) and was inactive at pH5.0 [20]. The previous low pH value was very useful in the process of enzyme extraction once it minimized the action of native pectin methylesterase, reduced the risk of microbial contamination, and stabilized pectin in solution [24, 25].

pH Stability

The effect of pH on the stability of *P. variotii* PG was investigated by incubating the enzyme at 25°C at different pHs for 24 h followed by dialysis for 1 h against 50 mM sodium acetate buffer, pH5.0 as the routinely used buffer. The results showed that the enzyme was very stable from pH3.0 to 6.0 and retained 95% of its activity until pH6.0. The enzyme lost about 26–35% of its activity from pH6.5 to 8.0 (Fig. 4b). The pH stability was also determined for PG from *Mucor flavus*, where the enzyme was completely stable between pH2.5 and 6.0 for 20 h at 20°C, but at pH7.0, the stability decreased to 60% [26]. Kobayashi et al. [19] reported that *Bacillus* sp. PG was very stable in various 50 mM buffers between pH6 and 12 when incubated at 30°C for 1 h.

Table 2 Relative activities of *P. variotii* PG toward substrates.

Substrates	% Relative activity
Sodium polypectate	100
Citric pectin (GM=9%)	67.6±2.3
Citric pectin (GM=30%)	50.1±1.7
Citric pectin (GM=70%)	33.2±0.9
Xylan	0.0±0.0
Starch	0.0±0.0
Glycogen	0.0±0.0

GM methyl group degree

Effect of Temperature on the P. variotii PG

P. variotii PG was found to have a temperature optimum at 65°C (Fig. 4c), where the hydrolase activity decreased 76.5% and 56.3% at 30 and 80°C, respectively. Similarly, temperature optimum for PG from *Thermoascus aurantiacus* CBMAI-756 [27] was approximately 60–65°C. On the other hand, temperature optima for PGs from *Bacillus* sp. [19], *Mucor flavus* [26], *T. reesei* [18], and *A. niger* NRRL3 [16] were approximately 40 and 50°C.

The temperature effect on thermal stability of *P. variotii* PG was investigated by incubating the enzyme up to 90 min in 50 mM sodium acetate buffer, pH5.0 at 45, 50, and 55°C prior to substrate addition. PG was totally stable at 45°C for 90 min. At 50°C, the activity decreased only 20% during the time of incubation. At 55°C the activity of the enzyme was gradually decreased in reference to time. While the enzyme retained 90% of its activity after incubation for 15 min, it retained 27% of activity after 90 min (Fig. 4d). The half-life time of the enzymatic activity was 50.6 min at 55°C. *T. reesei* PG1 and PG2 were

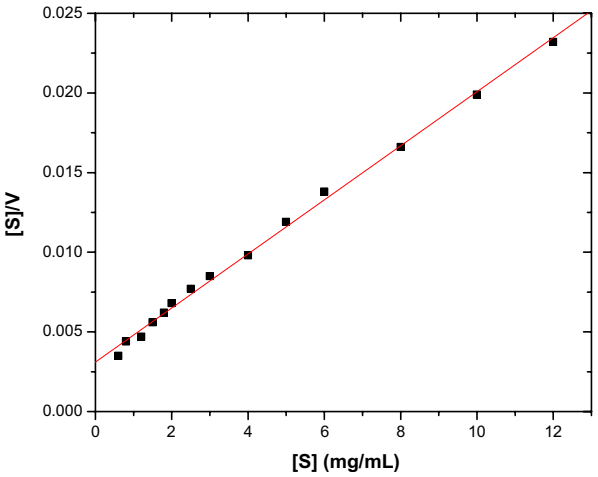

Fig. 5 Hanes–Woolf plot of *P. variotii* PG for PGA hydrolysis at pH4.0 and 65°C

stable up to 40 and 50 °C, respectively [18]. *Bacillus* sp. PG was stable up to 45 °C in absence of $CaCl_2$ and up to 50–55 °C in the presence of $CaCl_2$ [19]. The thermostability of the purified PG is higher than that of three isoforms of PG from mesophilic *Aspergillus* carbonarius (inhibited when incubated at 50 °C for 15 min) [28].

Substrate Specificity

A study of substrate hydrolysis for *P. variotii* PG was conducted by using pectins of different DEs (Table 2). The enzyme activities with different pectins were compared to the activity with NaPP, which was taken as 100%. The extent of hydrolysis of different pectins was decreased with an increase in DE. These relative rates were higher compared to *Bacillus* sp. PG, where citric pectins with a DE of 31%, DE 63%, and DE 93% had 42.7%, 8.3%, and 0.0% relative activity rates, respectively [19]. For characterization of PG with regard to substrate hydrolysis, other polysaccharides have been tested as substrates instead of pectins. The results showed that PG had no activity toward the examined polysaccharides.

Kinetic Parameters

The kinetic parameters of *P. variotii* PG for hydrolysis toward PGA at pH4.0 and 65 °C were obtained by Hanes plots (Fig. 5). The apparent K_m and V_{max} values for hydrolyzing NaPP were 1.84 mg/mL and 432 μmol/min/mg protein, respectively. Binding affinities (K_m) of the reported PGs using PGA ranged from 1.34 to 6.7 mg/mL [22, 28]. Using different pectins as substrates, the K_m of *A. niger* PGI ranged from 1.4 to 1.7 mg/mL. The varied K_m was also reported for PGs from *Neurospora crassa* (5.0 mg/mL) [29] and *Paenibacillus amylolytics* (4.6 mg/mL) [30]. From these results, it can be reported that *P. variotii* PG had high affinity (low K_m), using NaPP.

Table 3 Effect of metal ions on *P. variotii* PG.

Metal ions	% Relative activity	
	1 mM	10 mM
Control	100	100
Hg^{+2}	96.6±3.06	43.3±0.1
Zn^{+2}	96.3±0.2	13.9±0.34
Mn^{+2}	96.6±0.5	79.9±0.3
Fe^{+3}	86.1±1.2	50.5±1.6
Na^{+}	100±1.6	99.2±0.8
Mg^{+2}	98.3±2.8	67.4±0.2
Al^{+3}	99.3±0.01	15.0±2.1
Ag^{+2}	50.5±0.5	0.0±1.1
Pb^{+2}	100±1.2	86.1±0.5
K^{+}	100±1.4	94.3±1.2
Cu^{+2}	100±2.1	100±1.4
Co^{+2}	100±0.8	74.8±0.36

Effect of Metal Cations

The effect of different metal cations at the concentration of 1 and 10 mM on *P. variotii* PG assay system is shown in Table 3. Almost all the examined metal cations showed partial inhibitory effects under enzymatic activity, except for Na^{+1}, K^{+1}, and Co^{+2} (1 mM) and Cu^{+2} (1 and 10 mM). Several researchers have reported on the effect of metal cations on PGs. The activity of *Sporotrichum thermophile* Apinis PG was stimulated by Fe^{+2} and Mn^{+2} both 1 and 5 mM, while Ca^{+2} and Cu^{+2} stimulated only at 1 mM and inhibited at 5 mM. Mg^{+2} strongly inhibited enzyme activity [31]. The PG of *Bacillus* MG-CP-2 was stimulated by Ca^{+2} [32]. Addition of 0.01 mM $HgCl_2$ increased the PG2 activity of *A. niger* 3.4 times but did not affect PG1 [23].

Mode of Action of Exo-PG

The end products of enzymatic hydrolysis of NaPP were analyzed using thin layer chromatography (TLC). Galacturonic acid was the only degradation product detected. After increasing incubation times, no oligogalacturonates other than galacturonic acid were detected, which shows that the enzyme is specific in hydrolyzing the terminal glycosidic bond of the polymer, supporting the idea that it is an exo-PG (Fig. 6).

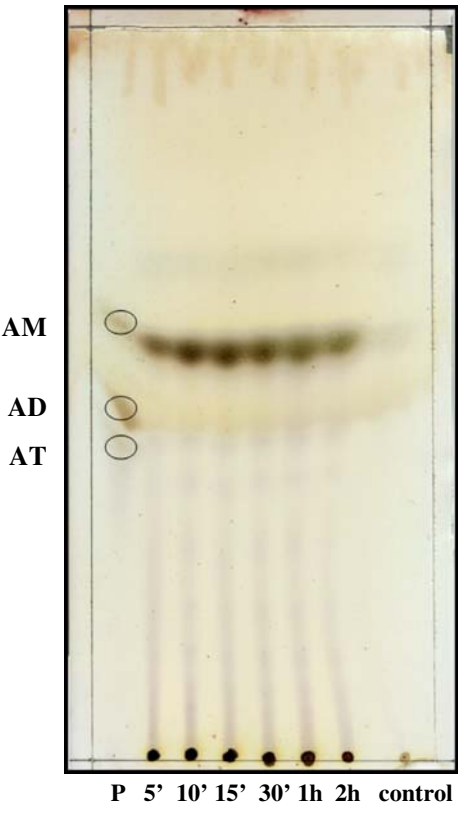

Fig. 6 TLC for analysis of NaPP hydrolysis products of PG from *P. variotii*. *AT* trigalacturonic acid, *AD* digalacturonic acid, *AM* galacturonic acid, *control* thermal inactive enzyme

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References

1. Gummadi, S. N., & Panda, T. (2003). Purification and biochemical properties of microbial pectinases–a review. *Process Biochemistry, 38*, 987–996. doi:10.1016/S0032-9592(02)00203-0.
2. Rexová-Benková, L., & Markovic, O. (1976). In R. S. Tipson & D. Horton (Eds.), *Advances in Carbohydrate Chemistry & Biochemistry* (pp. 323–385). New York: Academic.
3. Mohamed, S. A., Farid, N. M., Hossiny, E. N., & Bassuiny, R. I. (2006). Biochemical characterization of an extracellular polygalacturonase from *Trichoderma harzianum*. *Journal of Biotechnology, 127*(1), 54–64. doi:10.1016/j.jbiotec.2006.06.009.
4. Reid, I., & Ricard, M. (2000). Pectinase in papermaking solving retention problems in mechanical pulps bleached with hydrogen peroxide. *Enzyme and Microbial Technology, 26*, 115–123. doi:10.1016/S0141-0229(99)00131-3.
5. Wiseman, A. (1975). *Handbook of enzyme biotechnology* (p. 148). New York: Wiley.
6. Khanna, P., Sundari, S. S., & Kumar, N. J. (1995). Production, isolation and partial purification of xylanase from *Aspergillus* sp. *World Journal of Microbiology & Biotechnology, 11*, 242–243. doi:10.1007/BF00704661.
7. Rizzatti, A. C. S., Jorge, J. A., Terenzi, H. F., Rechia, C. G. V., & Polizeli, M. L. T. M. (2001). Purification and properties of a thermostable extracellular β-D-xylosidase produced by thermotolerant *Aspergillus phoenicis*. *Journal of Industrial Microbiology & Biotechnology, 26*, 156–160. doi:10.1038/sj.jim.7000107.
8. Adams, P. R. (1990). Mycelial amylase activities of thermophilic species of *Rhizomucor, Humicola* and *Papulaspora*. *Mycopatologia, 112*, 35–37. doi:10.1007/BF01795178.
9. Vogel, H. F. (1964). Distribution of lysine pathways among fungi: evolutionary implications. *American Naturalist, 98*, 435–446. doi:10.1086/282338.
10. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*, 426–429. doi:10.1021/ac60147a030.
11. Lowry, H., Rosebrough, N. J., Farr, A. L., & Randal, R. J. (1951). Protein measurement with the Folin phenol regent. *The Journal of Biological Chemistry, 193*, 267–275.
12. Davis, B. J. (1964). Disc electrophoresis II. Methods and application to human serum proteins. *Annals of the New York Academy of Sciences, 121*, 404–427. doi:10.1111/j.1749-6632.1964.tb14213.x.
13. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature, 227*, 680–685. doi:10.1038/227680a0.
14. O'Farrel, P. Z., Goodman, H. M., & O'Farrel, P. H. (1977). High resolution two dimensional electrophoresis of basic as well as acidic proteins. *Cell, 12*, 1133–1142. doi:10.1016/0092-8674(77)90176-3.
15. Hanes, C. S. (1932). The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *The Biochemical Journal, 26*, 1406–1421.
16. Fahmy, A. S., El-beih, F. M., Mohamed, A. S., Abdel-Gany, S. S., & Abd-Elbaky, E. A. (2008). Characterization of an Exopolygalacturonase from *Aspergillus niger*. *Applied Biochemistry and Biotechnology, 149*, 205–217. doi:10.1007/s12010-007-8107-x.
17. Pietro, A. D., & Roncero, M. I. G. (1996). Purification and characterization of an exo-polygalacturonase from the tomato vascular wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici*. *FEMS Microbiology Letters, 45*, 295–299. doi:10.1111/j.1574-6968.1996.tb08592.x.
18. Mohamed, S. A., Christensen, T. M. I. E., & Mikkelsen, J. D. (2003). New polygalacturonases from *Trichoderma reesei*: characterization and their specificities to partially methylated and acetylated pectins. *Carbohydrate Research, 338*, 515–524. doi:10.1016/S0008-6215(02)00398-1.
19. Kobayashi, T., Higaki, N., Suzumatsu, A., Sawada, K., Hagihara, H., Kawai, S., et al. (2001). Purification and properties of a high molecular-weight, alkaline exopolygalacturonase from strain of *Bacillus*. *Enzyme and Microbial Technology, 29*, 70–75. doi:10.1016/S0141-0229(01)00355-6.

20. Contreras-Esquivel, J. C., & Voget, C. E. (2004). Purification and partial characterization of an acidic polygalacturonase from *Aspergillus kawachii*. *Journal of Biotechnology, 110*, 21–28. doi:10.1016/j.jbiotec.2004.01.010.
21. Niture, S. K., & Pant, A. (2004). Purification and biochemical characterization of polygalacturonase II produced in semi-solid medium by a strain of *Fusarium moniliforme*. *Microbiological Research, 159*, 305–314. doi:10.1016/j.micres.2004.06.002.
22. Zhang, J., Bruton, B. D., & Biles, C. L. (1999). *Fusarium solani* endopolygalacturonase from decayed muskmelon fruit: purification and characterization. *Physiological and Molecular Plant Pathology, 54*, 171–186. doi:10.1006/pmpp.1998.0197.
23. Sakamoto, T., Bonnin, E., Quemerner, B., & Thibault, J.-F. (2002). Purification and characterization of two exo-polygalacturonases from *Aspergillus niger* able to degrade xylogalacturonan and acetylated homogalacturonan. *Biochimica et Biophysica Acta, 1572*, 10–18.
24. Gainvors, A., Nedjaoum, N., Gognies, S., Muzart, M., Nedjma, M., & Belarbi, A. (2000). Purification and characterization of acidic endo-polygalacturonase encoded by the PGL1-1 gene from Saccharomyces cerevisiae. *FEMS Microbiology Letters, 183*, 131–135. doi:10.1111/j.1574-6968.2000.tb08946.x.
25. Voragen, F., Beldman, G., & Schols, H. (2001). In B. V. McCleary & L. Prosky (Eds.), *Advanced dietary fibre technology* (pp. 379–397). Oxford: Blackwell.
26. Grade, R. V., Van Driessch, G., Van Beeumen, J., & Bhat, M. K. (2003). Purification, characterization and mode of action of an endopolygalacturonase from the psychrophilic fungus *Mucor flavus*. *Enzyme and Microbial Technology, 32*, 321–330. doi:10.1016/S0141-0229(02)00291-0.
27. Martins, E. S., Silva, D., Leite, R. S. R., & Gomes, E. (2007). Purification and characterization of polygalacturonase produced by thermophilic *Thermoascus aurantiascus* CBMAI-756 in submerged fermentation. *Antonie Van Leeuwenhoek, 91*, 291–299. doi:10.1007/s10482-006-9114-6.
28. Devi, N. A., & Rao, A. G. A. (1996). Fractionation, purification and preliminary characterization of polygalacturonases produced by *Aspergillus carbonarius*. *Enzyme and Microbial Technology, 18*, 59–65. doi:10.1016/0141-0229(96)00055-5.
29. Polizeli, M. L. T. M., Jorge, J. A., & Terenzi, H. F. (1991). Pectinase production by *Neurospora crassa*: purification and biochemical characterization of extracellular polygalacturonase activity. *The Journal of General and Applied Microbiology, 137*, 1815–1824.
30. Sakiyama, C. C. H., Paula, E. M., Pereira, P. C., Borges, A. C., & Silva, D. O. (2001). Characterization of pectin lyase produced by an endophytic strain isolated from coffee cherries. *Letters in Applied Microbiology, 53*, 117–221. doi:10.1046/j.1472-765x.2001.00961.x.
31. Kaur, G., Kumar, S., & Satyanarayana, T. (2004). Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile* apinis. *Bioresource Technology, 94*, 239–243. doi:10.1016/j.biortech.2003.05.003.
32. Kapoor, M., Beg, Q. K., Bhushan, B., Dadhich, K. S., & Hoondal, G. S. (2000). Production and partial purification of a thermo-alkali stable polygalacturonase from *Bacillus* sp. MG-CP-2. *Process Biochemistry, 36*, 467–473. doi:10.1016/S0032-9592(00)00238-7.