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Purification and characterization of an *exo*-polygalacturonase from *Pycnoporus sanguineus*

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ABSTRACT

The present work describes the purification and characterization of a novel extracellular polygalacturonase, PGase I, produced by *Pycnoporus sanguineus* when grown on citrus fruit pectin. This substrate gave enhanced enzyme production as compared to sucrose and lactose. PGase I is an exocellular enzyme releasing galacturonic acid as its principal hydrolysis product as determined by TLC and orcinol-sulphuric acid staining. Its capacity to hydrolyze digalacturonate identified PGase I as an *exo*-polygalacturonase. SDS-PAGE showed that PGase I is an *N*-glycosidated monomer. The enzyme has a molecular mass of 42 kDa, optimum pH 4.8 and stability between pH 3.8 and 8.0. A temperature optimum was observed at 50–60 °C, with some enzyme activity retained up to 80 °C. Its activation energy was 5.352 cal mol^{-1}. PGase I showed a higher affinity towards PGA than citric pectin ($Km = 0.55 \pm 0.02$ and 0.72 ± 0.02 mg ml^{-1}, respectively). Consequently, PGase I is an *exo*-PGase, EC 3.2.1.82.

Introduction

Plants are continually exposed to a vast array of potentially phytopathogenic microorganisms. The plant cell wall is a major barrier to the establishment of fungal infections. Enzymes capable of depolymerising components of the plant cuticle and cell wall are produced by fungal plant pathogens to aid their entry and spread within host tissue (Walton 1994). Most plant pathogenic fungi produce a number of cell wall-degrading enzymes (arabinosidases, xylosidases, glycosidases, pectinases) when grown in liquid media containing pectin (Yao *et al.* 1996). Pectins are very complex and heterogeneous molecules produced by all higher plants; they constitute a high proportion of the primary cell wall matrix and the middle lamella. Together with cellulose and hemicelluloses, they build the cell walls and contribute to many cell wall functions (Schols & Voragen 2002). Pectins are acidic polysaccharides that contain a homogalacturonan consisting of α-(1-4) linked-D-galacturonic acid residues and rhamnogalacturonans characterized by stretches of alternating α-(1-4) linked galacturonic acid and L-rhamnose and other monosaccharides. Besides their functions in living tissues, pectins are also of commercial interest, being used as gelling agents in the manufacture of jams and confectionery and for the

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stabilization of acidified dairy drinks (May 2000). In addition pectin fibres may be suitable for the preparation of various dietary products which could be useful in the prevention of hyperlipidemia as well as large bowel cancer (Kritchevsky & Bondfield 1997). Pectinases have potential applications in fruit, paper and textile industries. Apart from such industrial applications, these enzymes possess biological importance in protoplast fusion technology and plant pathology. Since applications of pectinases in various fields are widening, it is important to understand the nature and properties of these enzymes. There are two classes of PGases according to their mode of action on the polygalacturonic acid (PGA) chain. The pattern of degradation proceeds in either a random (*endo*-polygalacturonase EC 3.2.1.15) or terminal fashion (*exo*-polygalacturonases). Depending on their reaction products, enzymes that cleave homogalacturonanes in an exo-manner have been classified into two types, *exo*-polygalacturonases EC 3.2.1.67 and *exo*-polygalacturonases EC 3.2.1.82, which release monomeric or dimeric GalA, respectively (Parisot *et al.* 2003). Only limited information is available on the properties and reaction modes towards different substrates. In this work we isolated an *exo*-PGase produced by *Pycnoporus sanguineus* in liquid culture medium. The temperature and pH stability, molecular and kinetic characteristics were studied.

Materials and methods

Chemicals

All chemicals and reagents used were of the highest grade commercially available. Peptone, yeast extract and agar were provided by Difco (Detroit, Michigan, USA); Sephadex G-150 from Amersham Biosciences (Uppsala, Sweden); sodium acetate and acetic acid from Cicarelli Labs. (San Lorenzo, Sta. Fe, Argentina); Biogel P6 from BioRad Laboratories (Richmond, California, USA); Silicagel 60 plates from Merck (Damstadt, Germany). All other reagents were provided by Sigma–Aldrich (St. Louis, USA).

Organism

Pycnoporus sanguineus (L.: Fr.) Murr. was isolated from decaying wood and has been deposited as strain IEV 38 in the Culture Collection of the Institute of Plant Sciences, National University of Tucumán, Argentina. The strain is available on request.

Culture media

Agar containing 0.5 % (w/v) peptone, 1.5 % (w/v) yeast extract and 2.0 % (w/v) was used for maintaining *Pycnoporus sanguineus*. The same medium without agar was used as basic liquid medium (LM), augmented with 2 % sucrose, 2 % lactose or 1 % citric pectin. Two 5 mm diameter plugs of *P. sanguineus* mycelium were introduced into each 250-ml flask containing 100 ml autoclaved growth medium. Incubation was at 30 °C in the dark without agitation. Samples were taken every 7 d by filtering the contents of flasks through pre-weigh filter paper. Mycelium and filter paper were oven dried at 40 °C until constant weight. PGase activity was determined in the filtrate.

Extraction and PGase purification

LM with pectin as the only carbon source was inoculated with a plug of *Pycnoporus sanguineus* mycelium obtained from a fresh culture on SM. Mycelium grown in LM with 1 % citric pectin for 21 d in the dark at 30 °C was removed by filtration on Whatman N° 4 paper. Mycelium was washed twice with distilled water and disintegrated with a mortar and pestle in liquid nitrogen. The material was homogenized in 50 mM sodium phosphate buffer pH 7.2, containing 1 mM 2-mercaptoethanol (Buffer A). After centrifugation at 10 500 × g for 10 min, the supernatant was collected and dialyzed against 10 mM sodium acetate buffer pH 4.5 with 1 mM 2-mercaptoethanol (Buffer B). The dialyzed sample was kept at −20 °C until enzymatic activity was determined.

The culture filtrate was centrifuged at 10 500 × g for 15 min. From the supernatant (crude extract), proteins were concentrated by precipitation with solid ammonium sulphate between 30 and 80 % saturation. After centrifugation at 10 500 × g for 15 min, the precipitate was suspended in Buffer B and desalted overnight by dialysis against the same buffer. The dialysate was clarified by centrifugation at 10 500 × g for 10 min and the supernatant (4 ml) was applied to a Sephadex G-150 column (2.5 × 40.0 cm) equilibrated and eluted with buffer B. Eighty fractions of 2.5 ml were collected and absorbance at 280 nm and PGase activity were measured. Two peaks with PGase activity were detected (PGase I and PGase II). The most active fractions (PGase I) were combined and used for PGase characterization. All procedures were carried out at 4 °C. The enzyme preparation was kept at −20 °C until use for further assays.

Analytical methods

PGase activity was determined by measuring reducing sugar released from citric pectin or PGA by the Somogyi-Nelson method, using glucose as standard (Nelson 1944; Somogyi 1945). The standard reaction contained 0.1 ml aliquots of a suitable dilution of a purified enzyme preparation, 0.04 ml of 0.2 M sodium acetate buffer (pH 4.8) and 0.05 ml of 1 % (w/v) citric pectin or 1 % (w/v) PGA in a total volume of 0.25 ml. The mixture was incubated at 50 °C for 30 min and the reaction terminated by adding 0.5 ml of cuproalkaline reagent. After mixing, samples were immersed in a boiling water bath for 15 min and cooled in ice water to room temperature. Then, 0.5 ml of Nelson's reagent was added and the volume completed to 2.5 ml with distilled water. Reducing sugar formation was measured at 520 nm. Enzyme and substrate controls were assessed in triplicate for each sample. One unit (1 EU) of PGase activity was defined as the amount of enzyme required to release reducing sugars equivalent to 1 μmol glucose per min under the assay conditions. *Exo*-PGase activity was also measured using trigalacturonic acid (TGA) as the substrate instead of pectin or PGA.

The protein concentration was assayed either by Lowry's method (Lowry *et al.* 1951) using bovine serum albumin as the standard or by measurement of absorbance at 280 nm. Total neutral sugars were determined by the phenol-sulphuric acid method (Dubois *et al.* 1956) and expressed as glucose

equivalents. Three to five replicates were used for each sample determination.

Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing and denaturing polyacrylamide gel electrophoresis (PAGE and SDS-PAGE) were performed with a BioRad Mini Protean II system. For PAGE, purified PG I was run for 1 h at 100 V/plate in 7.5 % (w/v) polyacrylamide gel slabs at pH 8.8 (Davis, 1964). Gels were incubated for 15 min at room temperature in 50 mM sodium acetate buffer pH 4.8 containing 1.2 % (w/v) PGA. The gel was stained for 30 min with 0.02 % (w/v) ruthenium red and then destained by distilled water washes until polygalacturonase activity bands were visualized as clear areas over pink background (Ried & Collmer 1985; Lewosz 1993).

SDS-PAGE was performed according to Laemmli (1970). Purified PG I was heated at 100 °C for 5 min in the presence of 1 % (w/v) SDS and 10 mM 2-mercaptoethanol. Then, it was run for 1 h in 6 % (w/v) polyacrylamide gel slabs containing 0.1 % (w/v) SDS. Molecular mass markers (MW-SDS-70 Kit) were treated in the same way as samples and loaded onto the gel for calibrating the molecular mass of denatured PG I. The running conditions were 100 V, 45 mA/plate at a temperature of 4 °C. Proteins were stained by the silver nitrate method (Wray *et al.* 1981).

Analysis of hydrolysis products

Purified PGase I (0.015 units) was used to determine its hydrolysis products in a 0.2 ml reaction mixture using 0.1 M trigalacturonic acid (TGA), 1 % (w/v) citric pectin or 1 % (w/v) PGA as substrate. After incubation for an appropriate length of time (0–24 h), the mixture was heated at 100 °C for 30 min. A 20-μL sample from each incubation time was run on Silicagel 60 plates (20 × 20 cm). Twenty μL of 0.1 M monomer (GalA), 0.1 M trimer, 1 % pectin and polygalacturonic acid (PGA) were run as standards. Ascending TLC was developed with ethyl acetate/acetic acid/formic acid/water (9:3:1:4 v/v) as mobile phase. Galacturonic acids were detected by spraying the plate with 15 ml of 0.2 % (p/v) orcinol in sulphuric acid/methanol (1:9), followed by heating at 105 °C for 10–15 min until violet spots appeared on the plate (Fontana *et al.* 1988). Reduced TGA was prepared by treatment with 10 mM sodium borohydride ($NaBH_4$) in 25 mM NaOH at 30 °C for 4 h. Experiments were conducted twice with 2 replicates in each.

Molecular properties

The relative molecular mass of PGase I was estimated according to Andrews (1964) by gel filtration on Sephadex G-150 column and comparison with Molecular Weight Markers (MW GF-200). PGase I was deglycosylated by removal of N-linked carbohydrates with peptide *N*-glycosidase H. The reaction mixture consisted of 400 μl of PGase I (0.121 EU), 500 mU of peptide *N*-glycosidase H in 20 mM sodium phosphate buffer pH 6.8 with 20 mM EDTA, 1 mM sodium azide and 1 mM MPSF (methyl-phenyl-sulphonyl-fluoride) in 800 μL final volume. After incubation for 20 h at 37 °C the products were separated by Biogel P6 column (2 × 60 cm) using 10 mM sodium acetate buffer pH 4.8 with 0.2 M NaCl and 1 mM 2-mercaptoethanol (Buffer C). Two ml fractions were collected and Absorbance at 280 nm and neutral sugars were measured.

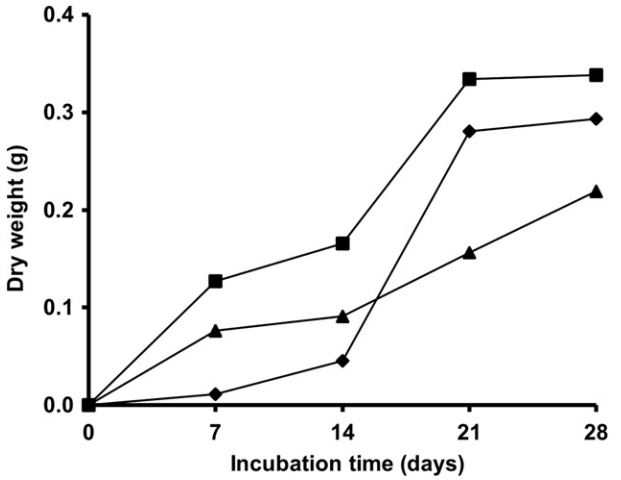

Fig 1 – *Pycnoporus sanguineus* growth in liquid medium with the carbon sources: pectin (■), sucrose (◆) and lactose (▲).

Enzyme catalytic properties

The activity profile of PGase I at various pH values (2.8–10.0) was determined at 50 °C. The pH stability was studied with

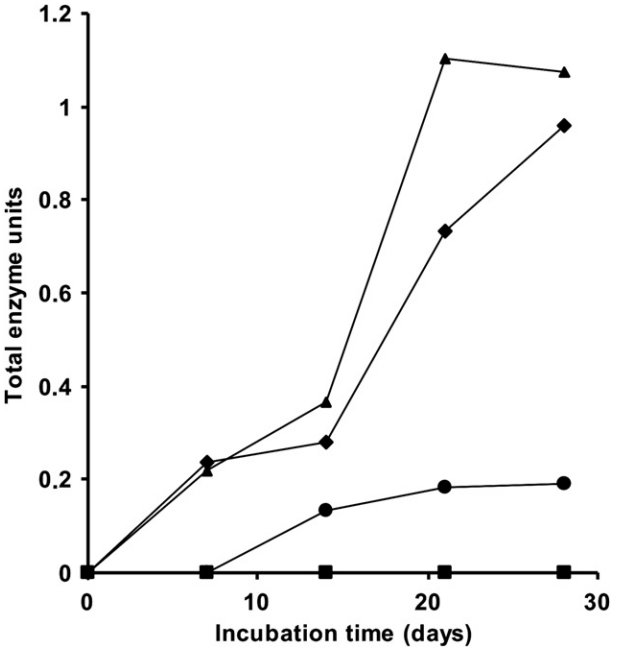

Fig 2 – Production of polygalacturonase by *Pycnoporus sanguineus* grown in liquid medium. Total enzyme units in the culture medium using pectin (▲) or sucrose (◆) as carbon source or in the mycelium grown on pectin (●) or sucrose (■) are indicated.

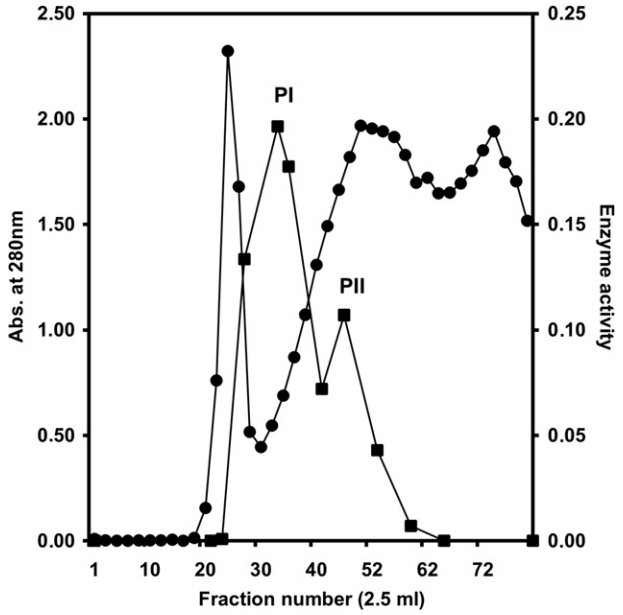

Fig 3 – Elution profile of a Sephadex G-150 gel filtration column to purify PGase from *Pycnoporus sanguineus*. The $(NH_4)_2SO_4$ precipitate fraction was loaded onto a column (40 × 2.5 cm) at a flow rate of 15 ml h^{-1}. Fractions were monitored spectrophotometrically at 280 nm (●) and for PGase activity (■).

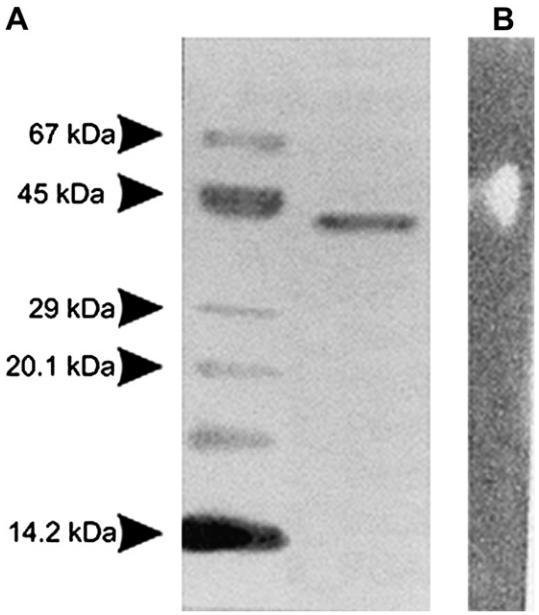

Fig 4 – (A) SDS-PAGE analysis of the Sephadex G-150 purified PGase I from *Pycnoporus sanguineus*. Proteins were stained with silver. Line 1, Mr markers; line 2, purified PGase I. (B) Activity staining of PGase I from *P. sanguineus* with ruthenium red after native PAGE analysis.

enzyme pre-incubated at 0 °C for 30 min at different pH (2.8–10.0). Thereafter, PGase I activity was determined at its optimal pH (4.8) at 50 °C. Thermal optimum activity was measured by incubating the enzyme at the optimum pH (4.8) at different temperatures (20–70 °C). The temperature stability profile of the enzyme was evaluated by measuring the residual activity after 30 min preincubation of the enzyme at temperatures ranging from 0 to 80 °C. Activation energy values were calculated with the Arrhenius equation (Maron & Prutton 1973).

Enzyme affinity towards citric pectin and PGA as substrates was investigated. The substrate concentration varied from 0.1 to 10 mg of substrate ml^{-1}. The Michaelis-Menten parameter (Km) and Vm were calculated from a double reciprocal plot of PG activity *vs* substrate concentration (Lineaweaver & Burk 1934).

Results

The total PGase activity produced by *Pycnoporus sanguineus* after 21 d growth on citric pectin was 2.3 and 3.0 times higher than that produced on sucrose or lactose, respectively. Fungal growth was also higher on pectin than sucrose or lactose (Fig 1). Therefore, citric pectin was used as the sole carbon source for further experiments. In these conditions *P. sanguineus* secreted *exo*-cellular PGases into the medium, whereas PGase activity was minimal or absent in the mycelial extract (Fig 2).

From 21-d old culture filtrates two PGases, PGase I and PGase II, were separated by precipitation with ammonium sulphate and gel filtration (Fig 3). PGase I had a higher activity and was therefore studied further. We purified this enzyme 200 times with a 54 % recovery. The specific PGase activity

Table 1 – Purification of the *exo*-polygalacturonase I of *Pycnoporus sanguineus*.

Purification step	Vol. (ml)	EU[a] ml^{-1}	Total EU	Proteins (mg ml^{-1})	Total proteins (mg)	Specific activity[b]	Purif.	Yield (%)
Crude preparation	300	0.0135	4.05	5.45	1635	2.5	1.00	100
Supernatant	290	0.0138	4.00	5.00	1450	2.7	1.08	99
$(NH_4)_2SO_4$ precipitation	8.6	0.3080	2.65	5.55	47.73	55.3	22.12	65
Sephadex G-150 fractionation	71	0.3100	2.20	0.62	4.40	500	200	54

a EU: enzyme units.
b Specific activity: total enzyme units 10^3 mg^{-1} of total proteins.

increased from 2.5 to 500 EU mg^{-1} of protein during the purification process, as summarized in Table 1.

The purity of pooled active PGase I fractions was evaluated by activity on a native PAGE gel and by SDS-PAGE stained for proteins. In both analyses, a single protein component was identified on the gel, indicating that PGase I was purified to apparent homogeneity (Fig 4A and B). The relative molecular mass of the enzyme was estimated to be approximately 42 000 by denatured PAGE. Similarly, the relative molecular mass of the native enzyme determined by gel permeation column chromatography was 42 300, indicating that the enzyme was a single polypeptide chain (Fig 4C).

To determine whether PGase I acted in an *endo* or *exo*-like manner, purified PGase I was incubated with pectin, polygalacturonic acid (PGA) and trigalacturonic acid (TGA) for different periods. The TLC analysis showed that the main end-product was the monomer (GalA). Increasing incubation times produced an increase of monomer and of medium-length oligo GAs (Fig 5). Moreover, the enzyme activity (crude extract) showed linearity with time up to 60 min and 400 μg of protein in the reaction mixture, indicating absence of dissociable activators or inhibitors in the extract. PGase I of *P. sanguineus* was a glycoprotein with a carbohydrate content of 23 % (w/w) (expressed as glucose equivalents). Furthermore, its glycosidic fraction was bound to nitrogen, as shown the treatment with *N*-glycosidase.

The activity of PGase I at its pH optimum (pH 4.8; Fig 6), increased from 20 to 60 °C, decreasing rapidly beyond. The highest enzymatic activity was between 50 and 60 °C (Fig 7), and the enzyme was stable between 0 and 60 °C. At 70 °C it lost 57 % of its hydrolytic activity, and only 10 % of the initial *exo*-PGase activity remained after 30 min of incubation at 80 °C. The Arrhenius plot showed an activation energy of 5352 cal mol^{-1}. The kinetic parameters of PGase I for hydrolysis towards citric pectin and PGA at pH 4.8 and 50 °C were obtained by a typical double reciprocal Lineweaver-Burk plots (Fig 8). The apparent Km values for hydrolyzing PGA and pectin were 0.55 ± 0.02 mg ml^{-1} and 0.72 ± 0.02 mg ml^{-1}, respectively. Values of Vm were 0.043 ± 0.019 μmol min^{-1} for PGA and 0.038 ± 0.019 μmol min^{-1} for citric pectin. Catalytic efficiency (Vm/Km) showed the enzyme to be more efficient for PGA than for citric pectin.

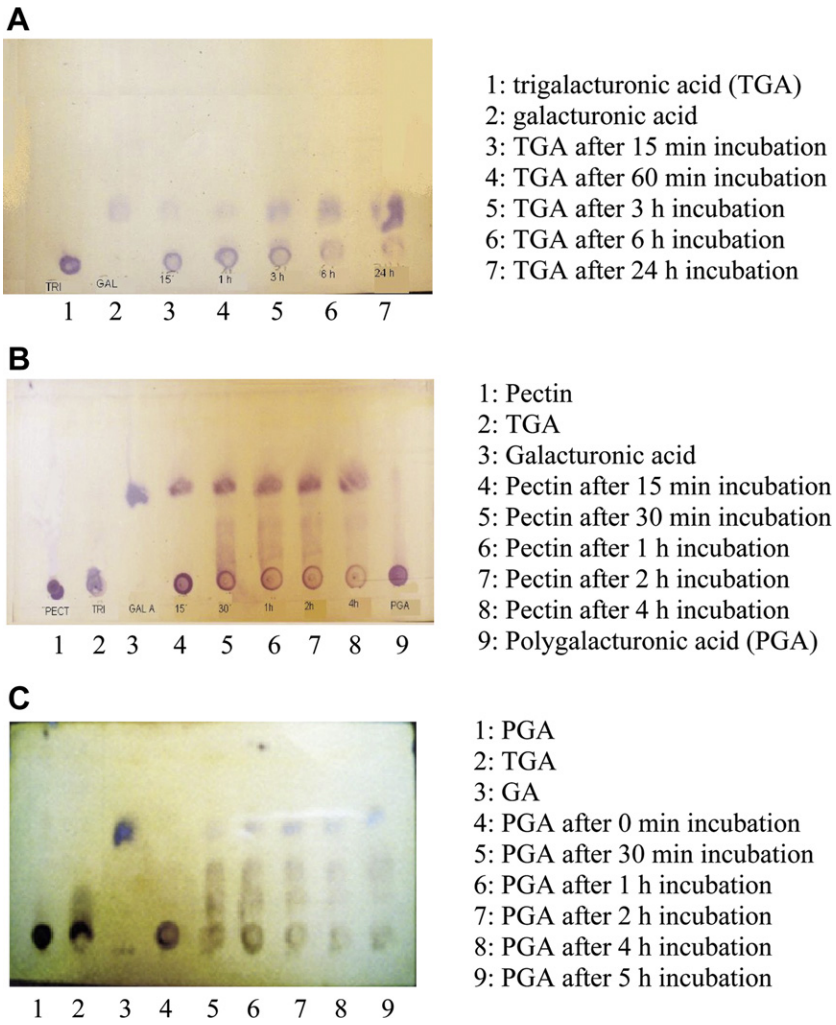

Fig 5 – Analysis of the PGase I product hydrolysis on various substrates after different incubation times. The substrates used were, (A) trigalacturonic acid; (B) citric pectin; (C) polygalacturonic acid.

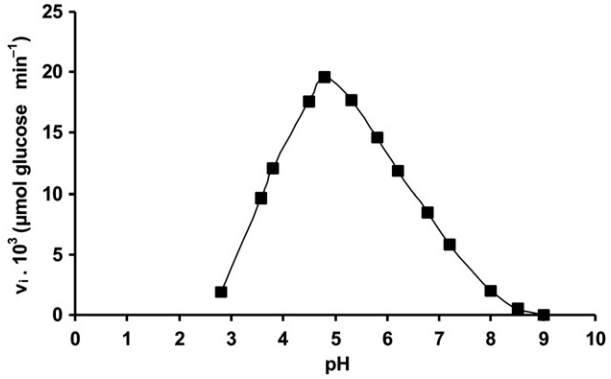

Fig 6 – Effect of pH on the reaction rate. All buffers used were 0.2 M. They were: HCl–glycine, pH 2.8–3.6; sodium acetate, pH 3.6–5.6; phosphate, pH 5.7–7.5; barbital/HCl, pH 8–10.

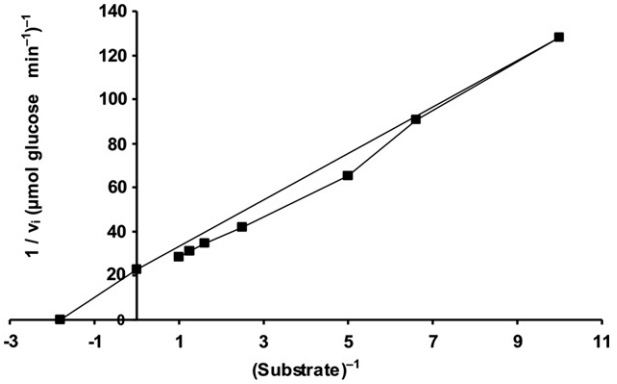

Fig 8 – Double reciprocal plot (Lineweaver Burk) of initial velocity against pectin concentration. Three replicates were used for each substrate concentration and the experiment was repeated three times.

Discussion

PGases show extensive variation in physical, chemical and kinetic properties. *Pycnoporus sanguineus* is considered as a low risk fungus by the American Type Culture Collection, justifying the studies on PGase production for diverse applications. The analysis of the fungal growth conditions indicated that the surface liquid culture was the most suitable from the point of view of the rate of fungal growth as well as the ease of mycelium separation, facilitating the process of PGase purification. Furthermore, the pH of the culture medium was constant during the fungal growth, providing an advantage for large-scale cultivation. The selected carbon source was citric pectin containing linear homogalacturonan regions of more than 100 GA residues.

High Mr's and an acidic optimum pH seem to be a general characteristic of fungal *exo*-PGases (Miyahiri *et al.* 2001; Santos *et al.* 2004; Jayani *et al.* 2005). Besides the high thermal stability of the enzyme from *P. sanguineus* is a useful characteristic for biotechnological applications. Analyses by TLC of the products of the action of PGase I from *P. sanguineus* on trigalacturonic acid, reduced trigalacturonic acid, citric pectin, and polygalacturonic acid as substrate, suggest that the purified PGase I behaved as an *exo*-polygalacturonase EC 3.2.1.67. This enzyme was a glycoprotein, a structural characteristic ascribed to transport, secretion and enzyme activity. The analyzed kinetic parameters (Km, Vmax) and the catalytic efficiency (Vmax/Km) demonstrated that PGase I of *P. sanguineus* is more efficient on PGA than on citric pectin degradation.

Such knowledge of the nature and properties of this enzyme is important for their efficient and effective use, since polygalacturonase applications are widening in several fields.

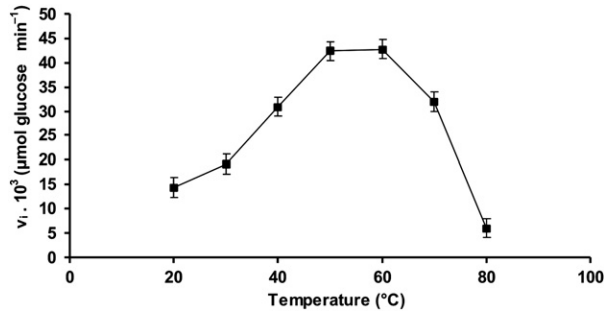

Fig 7 – Temperature effect on the reaction rate. Thermal optimal activity was measured by incubating the enzyme at the optimum pH (4.8) at different temperatures between 20 and 70 °C.

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