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ORIGINAL PAPER

Purification and characterization of the exopolygalacturonase produced by *Aspergillus giganteus* in submerged cultures

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Abstract Polygalacturonases are pectinolytic enzymes that catalyze the hydrolysis of the plant cell-wall pectin backbone. They are widely used in the food industry for juice extraction and clarification. *Aspergillus giganteus* produces one polygalacturonase (PG) on liquid Vogel medium with citrus pectin as the only carbon source. In specific applications, such as those used in the food and medicine industries, the PG must be free of substances that could affect the characteristics of the product and the process, such as color, flavor, toxicity, and inhibitors. We present here an efficient, simple, and inexpensive method for purifying the *A. giganteus* PG and describe the characteristics of the purified enzyme. Purified PG was obtained after two simple steps: (1) protein precipitation with 70% ammonium sulfate saturation and (2) anion-exchange chromatography on a DEAE-Sephadex A-50 column. The final enzyme solution retained 86.4% of its initial PG activity. The purified PG had a molecular weight of 69.7 kDa, exhibited maximal activity at pH 6.0 and 55–60°C, and was stable in neutral and alkaline media. It had a half-life of 115, 18, and 6 min at 40, 50 and 55°C, respectively. Purified PG showed its highest hydrolytic activity with low-esterified and nonesterified substrates, releasing monogalacturonic acid from substrate, indicating that it is an exopolygalacturonase. PG activity was enhanced in the presence of β-mercaptoethanol, dithiothreitol, Co^{2+}, Mn^{2+}, Mg^{2+}, NH_4^+, and Na^+ and was resistant to inhibition by Pb^{2+}.

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Introduction

Pectin, an important cell-wall component of dicotyledonous plants, is probably the most complex macromolecule in nature [26]. Pectin is responsible for the consistency, turbidity, and appearance of commercial fruit juices. The presence of pectic substances in fruit juices causes a considerable increase in their viscosity, thereby impeding the process of filtration and subsequent concentration during industrial-scale fruit juice processing [1].

Pectinolytic enzymes are naturally produced by many organisms, including bacteria, fungi, yeasts, insects, nematodes, protozoan, and plants. Microbial pectinases are important in the phytopathological process, in plant–microbe symbiosis, and in the decomposition of dead plant material, thereby contributing to the natural carbon cycle. Pectinases are abundantly produced by saprophytic fungi, and decaying plant tissue represents the most common substrate of pectinase-producing microorganisms [8].

Polygalacturonases (PGs) are the pectinolytic enzymes that catalyze the hydrolytic cleavage of the pectin chain with the introduction of water across the oxygen bridge. They have been most extensively studied in the family of pectinolytic enzymes [9]. PGs have been used on a wide scale in food processing industries for the extraction and clarification of fruit juices, especially those of the acidic fruits [1, 10].

An analysis of enzyme activity in the crude extract is unable to determine whether the activity is an isolated action or due to the presence of a multienzymic system

working in synergy on the degradation of the substrate. The characterization of purified enzymes is an important area of research since it focuses on being able to distinguish between the enzymatic complex components of the substrate degradation mechanism, optimum conditions for enzymatic activity, and the regulation of enzyme synthesis [8, 21]. In addition, the use of enzymes in such industries as food processing and medicine production requires that the enzyme being used is a pure preparation in order to guarantee the quality of the final product and to avoid color and flavor changes due to contaminants in the enzyme extract itself. A pure preparation will also eliminate substances that could act as inhibitors of the enzyme activity and toxins.

Aspergillus giganteus, an imperfect ascomycete, is known to secrete an antifungal protein (AFP) which is a small basic protein that inhibits the growth of several filamentous fungi, mainly from genera *Fusarium* and *Aspergillus* [25]. The strain used in the study reported here has been studied in our laboratory for pectinase production. It is able to produce high levels of pectin lyase and PG. The highest amount of PG is obtained when the strain is grown in liquid medium, pH 3.5, containing ammonium sulfate as the nitrogen source and citrus pectin as the carbon source and shaken at 120 rpm for 4.5 days at 30°C [20]. We report here an efficient, simple, and cheap method for purifying *A. giganteus* PG and describe the main characteristics of the purified enzyme required for its industrial application.

Materials and methods

Culture and maintenance of fungal strain

Aspergillus giganteus strain CCT 3232 is maintained in the culture collection of the "Fundação Tropical de Pesquisa e Tecnologia André Tosello" (Brazil). It was previously isolated from the soil of the Brazilian Atlantic Forest, at Peruíbe, São Paulo State. The fungus was maintained on Vogel medium [27] agar slopes. The inoculum preparation and enzyme production were carried out as described earlier [20].

Polygalacturonase (PG) activity

For assaying enzyme activity, the enzyme solution was first with polygalacturonic acid in imidazole–NaOH, 50 mM, pH 6.0, at 55°C. The reaction was stopped by adding dinitrosalicylic acid reagent (DNS) at 10 and 20 min into the reacion, and the unhydrolyzed substrate was removed by centrifugation. Reducing sugars were then measured in the supernatant by Miller's method [14], using galacturonic acid as the standard. One unit of activity was defined as the amount of enzyme releasing 1 μmol of reducing groups per minute under the experimental conditions. Specific activity was based on the concentration of extracellular protein in the enzyme solution.

Protein determination

Protein content was measured by the Lowry [13] method using bovine serum albumin as the standard or by measuring absorption at 280 nm.

Enzyme purification

Ammonium sulfate was added to the culture extract up to 70% saturation; the insoluble contents were then removed by centrifugation (10,000 *g*, 20 min). The supernatant was dialyzed against Tris-HCl 50 mM, pH 8.0, at 4°C. The dialyzed sample was applied to a DEAE-Sephadex A-50 column (17 × 2.2 cm; Pharmacia AB, Stockholm, Sweden) equilibrated with the same buffer, at a flow rate of 48 ml h^{-1}. The column was washed with the starting buffer, and the bound protein was then eluted with a linear gradient of NaCl (0.0–0.5 M). Fractions of 3.0 ml were collected, and those exhibiting PG activity were pooled.

Enzyme characterization

Influence of pH and temperature The influence of pH on PG activity was measured by incubating the purified enzyme with the substrate at 55°C at various pH values between 4 and 8. Three buffers were used, namely, 0.05 M sodium acetate buffer from pH 4.0–5.5; 0.05 M sodium imidazole buffer for pH 6.0–6.5; 0.05 M Tris-HCl buffer from pH 7.0–9.0. To determine the optimal temperature for PG activity, the enzyme was incubated with the substrate at pH 6.0, at various temperatures between 35 and 75°C.

Enzyme stability The thermal stability of PG activity was assayed as residual activity after incubating the culture filtrate with sodium imidazole buffer, pH 6.0, at 40, 50, and 55°C, without substrate. The pH stability was determined as the residual PG activity after the purified enzyme had been incubated for 24 h at 4°C, without substrate, in the buffers 0.05 M glycine-HCl (pH 3.0–3.5), 0.05 M sodium acetate (pH 4.0–5.5), 0.05 M sodium imidazole (pH 6.0–6.5), 0.05 M Tris-HCI (pH 7.0–9.0), or 0.05 M glycine-NaOH (pH 9.5–10.0).

Analytical electrophoresis Homogeneity and the molecular mass of the purified PG was checked by electrophoresis on 8–18% polyacrylamide gels (sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [12]. Protein bands in the gels were stained with Coomassie brilliant blue G-250. The second

determination of molecular mass was performed by loading the enzyme on a Sephadex G-100 column (64 × 2.6 cm).

Thin layer chromatography For the thin layer chromatography (TLC) analysis of polygalacturonic acid and citrus pectin degradation products, heat-inactivated samples were spotted on silica gel 60 aluminum sheets (15 × 10 cm; Merck, Germany), and the chromatography was performed twice using the ascending method with ethyl acetate:acetic acid:formic acid:water (9:3:1:4) as the solvent system. For visualization of the spots, the dried plate was sprayed with 0.2% orcinol dissolved in 10% sulfuric acid in methanol followed by heating at 105°C for 5 min.

Substrate specificity Substrate specificity of the enzyme was studied by incubating the purified PG, at the optimum pH and temperature, with different substrates (citrus pectin with three different methylation degrees of the carboxyl groups—34, 72 and 90%; apple pectin; polygalacturonic acid) at a fixed concentration (1%).

Enzyme kinetics The kinetic constants K_m, V_{max}, and k_{cat} of the enzyme were calculated by fitting the activity data at different substrate concentrations to a linear regression on Lineweaver–Burk double-reciprocal plots.

Influence of metal ions and reagents on enzyme activity The effect of a number of cations, a denaturant, and chelating agents on enzyme activity was tested in the reaction medium. PG was assayed in the presence of various substances at 2 and 10 mM, or at 2 and 5 mM for substances that caused over-gelatinization of the substrate (polygalacturonic acid) at 10 mM. Before the assay, the enzyme solution was dialyzed against 0.05 M imidazole buffer, pH 6.0.

Results

Isolation of polygalacturonase

Homogenous PG was obtained using a two-step procedure: (1) precipitation of proteins with 70% ammonium sulfate saturation; (2) anion-exchange chromatography on a DEAE-Sephadex A-50 column (Fig. 1). Following the addition of ammonium sulfate, almost all PG activity was recovered in the supernatant with a purification fold of 4.0 (Table 1). The PG solution was dialyzed, applied to an anion-exchange column, and eluted with a linear salt gradient. The active fractions were pooled, and the sample showed a final purification fold of 28.1 and a high yield of 86.4% (Table 1).

Most of the methods for purifying fungal and bacterial PGs that have been published to date are associated with undesirable enzyme losses during the purification process, with the recovery level being, in most cases, <50% [2, 4, 11, 22, 24] and, occasionally, <1% [3, 18]. These considerable enzyme losses are mainly caused by the relatively high number of steps required in the purification process which, in turn, also increase the cost of the purification process. Consequently, many purification processes are economically unattractive for application on the industrial scale.

The two-step method for *A. giganteus* PG purification described here has, therefore, a great potential value for producing PG on an industrial scale since can achieve high enzyme yields after two simple steps.

A. giganteus produced only one PG in the presence of citrus pectin as an inducer, which is not common. Fungi usually produce a set of pectinolytic enzymes, including two or more PG isoforms [2, 5, 15, 24].

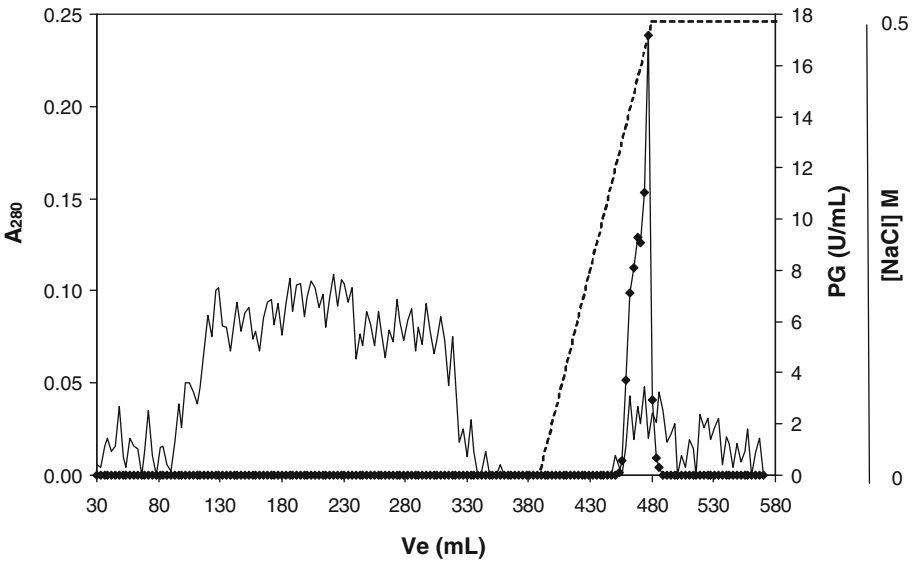

Fig. 1 Anion-exchange chromatography on DEAE-Sephadex A-50 column of polygalacturonase (*PG*) produced by *Aspergillus giganteus*. *Solid line* Absorbance at 280 nm, *filled diamonds* PG activity, *broken line* salt gradient

Table 1 Polygalacturonase purification from *Aspergillus giganteus*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	242.7	16.3	14.9	100.0	1.0
Amonium sulfate precipitation	241.5	4.0	60.4	99.5	4.0
DEAE-Sephadex column	209.7	0.5	419.4	86.4	28.1

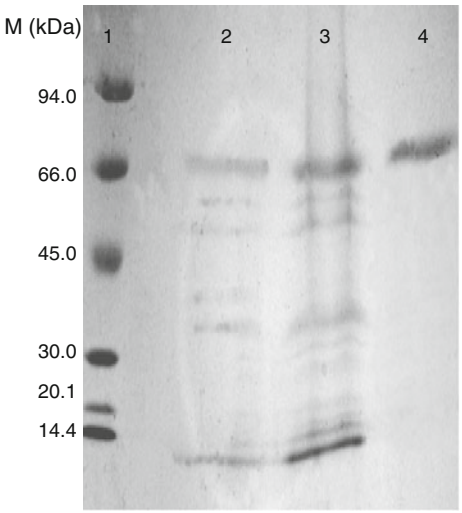

Fig. 2 Analysis of PG purification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE-SDS). *Lanes*: *1* Molecular mass standard proteins, *2* supernatant sample after protein precipitation, *3* crude extract, *4* sample from DEAE-Sephadex column

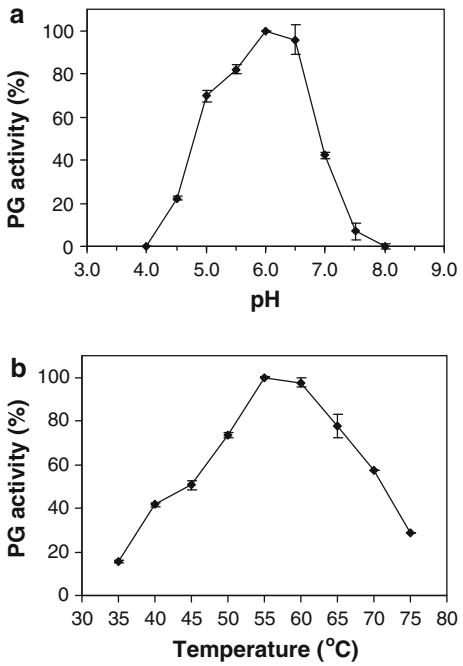

Fig. 3 Influence of pH (**a**) and temperature (**b**) on purified PG activity from *A. giganteus*. For both assays the *vertical bars* Standard deviation (SD) of the mean calculated for three replicates

Main properties of the purified polygalacturonase

The purified PG had a molecular mass of 69.7 kDa as determined by SDS–PAGE (Fig. 2) and 69.8 kDa as determined by gel filtration. These are higher values than those reported to PGs from other sources, which usually range between 40 and 60 kDa [9].

The dependence of enzymatic activity of the isolated enzyme on pH is shown in Fig. 3a. The enzyme was active in acid and neutral medium, exhibiting optimal activity at pH 6.0–6.5. Most of the fungal PGs described in the literature have maximal activity at a pH range of 4.0–5.0 [9, 17]. Most PGs are produced by fungi, and fungal PGs are more active in acid or neutral medium [21].

In terms of temperature, purified PG showed maximal activity between 55 and 60°C (Fig. 3b). Fungal PGs frequently show optimal activity temperatures between 40 and 60°C [21].

The thermal stability of the purified PG was assayed immediately after its incubation without substrate at three different temperatures. The half-life of PG at 40, 50, and 55°C was 115, 18, and 6 min, respectively (Fig. 4a). The purified PG from *A. giganteus* is therefore more heat-tolerant than PG I and II from *A. japonicus*, both of which were found to lose ≥50% of their activity during a 5-min incubation at 50°C [24]. The enzyme has been shown to lose stability after purification, with a half-life of 90 min at 55°C in the crude extract [20]. Ortega et al [19] observed the same stability decrease after PG purification. This change in stability can be attributed to the thermo-protective effect of impurities.

The isolated PG proved to be very stable over a neutral and alkaline pH range, retaining ≥95% of its activity after 24 h at any pH between 6.5 and 10.0 (Fig. 4b). Nevertheless, fungal PGs are usually stable only in acid medium [5, 6, 9, 18]. The PGs from *Aspergillus kawakii* [4] and *Rizopus oryzae* [22] maintained ≥90% of their activity only when incubated at pH 5.0 and 5.5 (24 h at 25°C), respectively. The purified PG from *Mucor flavus* retained ≥90% of its activity at pH 2.5 and 6.0 when incubated for 20 h at 20°C [7].

The enzyme was active towards substrates with any degree of methyl-esterification (0, 34, 72, 75, and 90%) and was better at hydrolyzing low-esterified and nonesterified

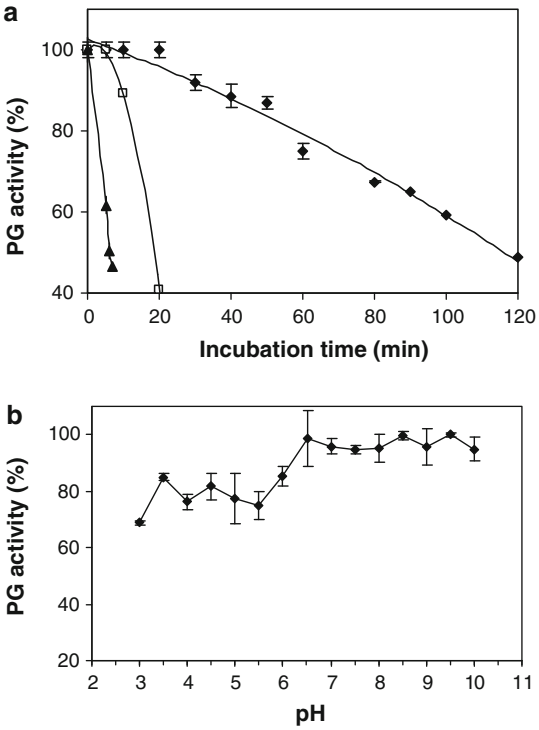

Fig. 4 Thermal inactivation (**a**) and pH stability (**b**) of purified PG from *A. giganteus*. Temperatures: *filled triangle* 55°C; *square* 50°C, *filled diamond* 40°C. For both assays the *vertical bars* SD of the mean calculated for three replicates

Table 2 Substrate specificity of PG activity from *A. giganteus*

Substrate	PG (U ml^{-1})	PG (%)
Citrus pectin (MD 34%)	7.20 ± 0.2	100 ± 2.8
Polygalacturonic acid	6.83 ± 0.2	94.9 ± 2.8
Citrus pectin (MD 72%)	3.74 ± 0.3	51.9 ± 4.2
Citrus pectin (MD 90%)	1.91 ± 0.1	25.5 ± 1.4
Apple pectin (MD 75%)	1.72 ± 0.06	23.9 ± 0.8

MD, Methylation degree of the carboxyl groups of pectin

The results are given as the mean calculated for three replicates ± standard deviation (SD)

substrates (Table 2), showing a V_{max} of 669.6 and 602.8 μmol mg^{-1} min^{-1} and a K_m of 3.25 and 1.16 mg ml^{-1} with 34% esterified citrus pectin and polygalacturonic acid as substrates, respectively (Table 3). The K_m values indicate that PG from *A. giganteus* has a high affinity for the nonesterified substrate polygalacturonic acid. Those results validate the enzyme classification as a PG. Purified PG showed a higher turnover acting on citrus pectin than on polygalacturonic acid (k_{cat} 770 and 690 s^{-1}); however, the catalytic efficiency value was 2.5-fold higher for the latter (Table 3).

The only soluble product released in the hydrolysis of pectin and polygalacturonic acid was monogalacturonic

Table 3 Kinetic parameters of PG from *A. giganteus*

Kinetic parameters	Polygalacturonic acid	Citrus pectin (MD 34%)
V_{max} (μmol ml^{-1} min^{-1})	8.7	9.7
V_{max} (μmol mg^{-1} min^{-1})	602.8	669.6
K_m (mg ml^{-1})	1.16	3.25
k_{cat} (s^{-1})	690	770
k_{cat}/K_m (mM^{-1} s^{-1})	126.6	50.3

Values are given as the mean calculated for three replicates

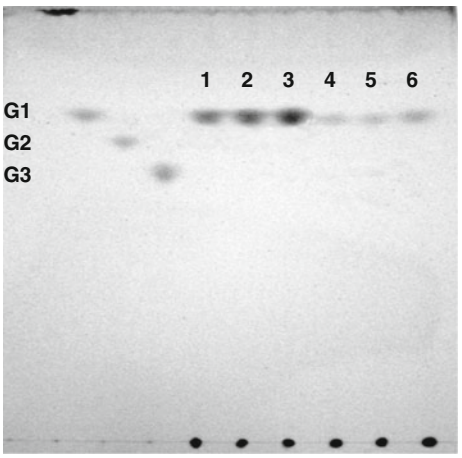

Fig. 5 Hydrolysis pattern of polygalacturonic acid (*1*, *2*, *3*) and low methylesterified citrus pectin (*4*, *5*, *6*) by the purified PG from *A. giganteus*. Each substrate was incubated in the presence of the enzyme for 30 min (*1*, *4*), 2 h (*2*, *5*) and 20 h (*3*, *6*). Standards: *G1* monogalacturonic acid, *G2* digalacturonic acid, *G3* trigalacturonic acid

acid (Fig. 5). Based on these results the enzyme can be classified as exopolygalacturonase (EC 3.2.1.67). Endo-PGs are widely distributed among fungi, bacteria, and many yeasts and are also found in higher plants and some plant parasitic nematodes. In contrast, exo-PGs occur less frequently, being reported mainly in bacteria. Exo-PGs can be classified into two types: fungal exo-PGs, which produce monogalacturonic acid as the main end product, and the bacterial exo-PGs, which produce digalacturonic acid as the main end product [9]. The PG from *A. giganteus* is a typical fungal exo-PG.

Since pectic substrates are susceptible to undergoing changes in their physical and chemical properties changes in the presence of salts and other substances, the effect of a number of cations, a denaturant, and chelating agents on enzyme activity were tested in the reaction medium. PG was assayed in the presence of various substances at 2 and 10 mM, or at 2 and 5 mM for those substances that caused over-gelatinization of the substrate (polygalacturonic acid) at 10 mM. Before the assay, the enzyme solution was dialyzed against 0.05 M imidazole buffer, pH 6.0. Purified

Table 4 Influence of substances on polygalacturonase activity from *A. giganteus*

Substance	PG activity (%)[a,b]	
	2 mM	10 mM
β-Mercaptoethanol	142.6 ± 2.6	146.1 ± 0.4
Dithiothreitol	100 ± 0.4	135.2 ± 7.9
$MgSO_4$	112.3 ± 1.5	120.2 ± 8.0
NH_4Cl	114.9 ± 4.0	107.7 ± 6.1
NaCl	100 ± 2.2	114.3 ± 1.3
$CaCl_2$	100 ± 2.3	94.7 ± 5.2
Sodium dodecyl sulfate	102.8 ± 6.2	79.1 ± 2.6
Iodoacetic acid	101.8 ± 8.3	66.1 ± 0.2
Phenylmethanesulfonylfluoride	89.7 ± 3.5	25.4 ± 1.1
Sodium citrate	82.5 ± 4.0	71.6 ± 0.9
Ethylenediaminetetraacetic acid	77.2 ± 3.5	21.5 ± 0.4
$HgCl_2$[b]	0	0
Substance	2 mM	5 mM
$MnSO_4$	114.9 ± 8.4	132.5 ± 1.1
$CoCl_2$	120.9 ± 3.5	119.0 ± 0.8
$ZnSO_4$	102.8 ± 9.7	88.5 ± 1.0
$BaCl_2$	91.9 ± 7.5	86.3 ± 0.4
$Pb(CH_3COO)_2$	89.7 ± 1.8	87.5 ± 6.6
$CuCl_2$	49.2 ± 6.1	48.2 ± 1.3

Values are given as the mean calculated for three replicates ± SD

[a] Control: 100 ± 5.7%

[b] Activity buffer: sodium acetate 0.05 M pH 5.5

PG activity was strongly stimulated in the presence of β-mercaptoethanol (2 and 10 mM) and dithiothreitol (DTT; 10 mM), while phenylmethanesulfonylfluoride (PMSF; 2 and 10 mM) and iodoacetic acid (10 mM) inhibited enzyme activity (Table 4). These results suggest a critical role of the cysteine residue(s) in the catalysis and/or substrate binding by the *A. giganteus* exo-PG reaction [16, 23]. The cations Co^{2+}, Mn^{2+}, Mg^{2+}, and NH_4^+ at both concentrations and Na^+ at 10 mM enhanced enzyme activity, but Ca^{2+} did not show any significant effect on PG activity. A slight inhibition of purified PG activity was observed in the presence of Zn^{2+} (5 mM), Ba^{2+}, and Pb^{2+} (2 and 5 mM). Purified PG activity was inhibited by Cu^{2+} and completely lost in the presence of Hg^{2+}. Heavy metal ions, such as Hg^{2+}, in reaction medium can activate oxygen molecules, accelerating the oxidation of thiol groups [23], suggesting again a critical role of cysteine residue(s) in the catalysis. The protein denaturant SDS affected PG activity only at high concentrations. The chelating agents ethylenediaminetetraacetic acid (EDTA) and citrate also inhibited the activity, indicating an important role of free cations in the catalysis process and/or in the maintenance of the native enzyme three-dimensional structure.

Conclusion

The exopolygalacturonase from *A. giganteus* was efficiently purified using a two-step procedure with a high recovery. The enzyme showed interesting characteristics, such as a maximum activity in slight acid and neutral medium as well as a high stability over a large pH range and resistance to heavy metals (Mn^{2+} and Pb^{2+}). These properties suggest that the enzyme has potential applications in various food industries in general and in juice and wine production and vegetable maceration processes in particular. *A. giganteus* can be considered a good alternative for pectinase production on an industrial scale since it is a good producer, and the purifying process described here is totally feasible for large-scale PG purification since it is efficient, inexpensive, and simple; it also allows for enzyme recovery at a high level.

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