

Purification and Characterization of a Polygalacturonase Produced by *Wickerhamomyces anomalus*

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ABSTRACT

The aim of this work was to study the purification and physicochemical properties of an endo-polygalacturonase (PG) produced by *Wickerhamomyces anomalus* isolated from the citrus fruit peels. The enzyme was purified to homogeneity from the culture filtrate of *W. anomalus* grown on the yeast nitrogen base medium with glucose as carbon and energy source and citrus pectin as inductor. After anion-exchange chromatography and gel filtration chromatography, PG activity was eluted as a single peak, yielding 21% of the original activity. After dialysis and cation-exchange chromatography, only one fraction with PG activity was obtained, recovering 56% of initial enzyme activity and 1.3-fold increase in specific activity. The molecular weight of the enzyme was estimated as 43 kDa by the SDS-PAGE. The enzyme exhibited maximal activity at pH 4.2 and was stable over a pH range from 3.5 to 6.0 and up to 49°C for 10 h. The V_{max} and K_m values with polygalacturonic acid as substrate were 0.26 mmol/L.min and 0.173 mg/mL, respectively. Cations such as Cu^{+2} , Fe^{+3} , Mg^{+2} , Mn^{+2} and Zn^{+2} did not show any significant effect on PG activity but K^+ and Ca^{+2} reduced it. The purified PG was able to macerate cassava tissues.

Key words: *Wickerhamomyces anomalus*, polygalacturonase, purification, characterization

INTRODUCTION

Enzymes hydrolyzing the pectic substances are known as pectinolytic enzymes or pectinases. These enzymes are responsible for the degradation of pectic substances that occur as structural polysaccharides in the middle lamella and the primary cell walls of plant tissues. Based on their mode of action, these enzymes are classified as polygalacturonase (PG), pectin esterase (PE), pectin lyase (PL) and pectate lyase (PAL). PG, PL and PAL are depolymerizing enzymes, which split the α -(1,4)-glycosidic bonds between galacturonic monomers in pectic substances either by the hydrolysis (PG) or by β -elimination (PL, PAL). PE

catalyzes the de-esterification of the methoxyl group of pectin, forming pectic acid (Jayani et al. 2005; Tari et al. 2007).

Pectinases play a key role in food technology, mainly in the processing of fruit juices and wines and in the maceration of plant tissues (Croak and Corredig 2006; Nighojkar et al. 2006). Maceration is a process by which organized tissue is transformed into a suspension of intact cells by the action of pectinases, resulting in pulpy products used for nectar as baby foods and ingredient for dairy products. For such purposes, only the intercellular cementing material that holds together the cells (protopectin) and some portion of primary plant cell walls should be removed without

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damage to adjacent secondary cell walls, to help avoid cell lysis, keeping the nutritional properties of food (Costa et al. 2007).

A pectinolytic yeast strain was isolated from the citrus fruit peels in the province of Misiones, Argentina and it was identified as *Wickerhamomyces anomalus*, re-classification of the species *Pichia anomala* (Kurtzman 2008; Martos et al. 2013a). This wild yeast strain, grown on a yeast nitrogen base medium with glucose and citrus pectin produced an endo-PG with maceration activity of vegetable tissues. The supernatant of *W. anomalus* was able to macerate cassava and potato tissues (Martos et al. 2013a, b). In view of the potential applicability of PG secreted by *W. anomalus* in food technology, the present work reports the purification and some relevant physicochemical properties of the enzyme.

MATERIALS AND METHODS

Microorganism

W. anomalus, isolated from the citrus fruit peels in the province of Misiones, Argentina (Martos et al. 2013a) was used in this study.

Culture media

YM medium contained (g/L) yeast extract (Sigma Chemical Co., St. Louis, Mo, USA) 5, tryptone (Difco-Becton Dickinson & Co., Sparks, MD, USA) 5, glucose (Britania, Buenos Aires, Argentina) 10, agar (Britania), 15 and the pH was 5.0.

YNB medium contained (g/L) yeast nitrogen base (YNB, Difco) 6.7, glucose (Britania) 5, citrus pectin (Parafarm, Buenos Aires, Argentina) 5 and the pH was 5.0. Citrus pectin was washed with 70% (v/v) ethanol-HCl (0.05 N) solution to remove the soluble sugars (Cavalitto et al. 1996). All the components of media were autoclaved (121°C, 15 min), except in the case of YNB solution, which was sterilized separately by filtration through a cellulosic filter paper (0.22 µm, Sartorius).

Enzyme production

Five hundred millilitre Erlenmeyer flasks with 95 mL of YNB medium were inoculated with 5.0 mL of an appropriate dilution of a suspension of the microorganism ($OD_{620} = 0.96$), grown in YM medium (30°C, 24 h). The Erlenmeyer flasks were

incubated at 30°C for 10 h on a rotary shaker at 180 rpm. The biomass was separated by centrifugation at $2350 \times g$ at 5°C for 10 min. The culture medium supernatant, named enzymatic extract (EE), was frozen at -18°C and used as source of extracellular enzyme (Martos et al. 2013a).

Enzyme purification procedures

The EE (500 mL) was concentrated ($2 \times$) on a vacuum rotary evaporator at 38°C. The resulting solution was lyophilized to dryness and re-suspended ($5 \times$) in sodium acetate buffer (AcB 0.2 M, pH 5.0). The concentrated enzymatic extract (CEE) obtained was subject to two purification procedures (Procedure I and Procedure II). All the chromatographic steps were carried out on an Amersham FPLC system (ÄKTA FPLC-U900 Chromatographic System, GE Healthcare). After each chromatographic step, fractions with PG activity were applied to a PD-10 desalting column (GE Healthcare) and then loaded onto a single well 10% SDS-PAGE minigel, in a Mighty Small II Unit (Hofer SE 260, GE). Proteins were visualized by Coomassie Brilliant Blue G-250 staining (Smith 1984).

Procedure I

The CEE was desalted on a Sephadex G-25 gel filtration column (XK 16/100, GE Healthcare), equilibrated with AcB (0.2 M, pH 5.0). The column was eluted with the same buffer over 3.4 column volumes, at a flow rate of 2 mL/min, during 1.5 h. Fractions of 3.0 mL were collected and those exhibiting PG activity were pooled, freeze-dried, re-suspended in AcB and poured over a Q Sepharose FF anion-exchange column (XK 26/20, GE Healthcare). The column was washed with AcB and the bound proteins were then eluted with a linear gradient of NaCl (0–0.5 M) in the same buffer over 7.5 column volumes at a flow rate of 1.5 mL/min for 3 h. Fractions (3.0 mL) were collected and analyzed for PG activity. Fractions with PG activity were pooled, concentrated by lyophilization and applied to a PD-10 desalting column (GE Healthcare) equilibrated with the same buffer. The eluate was subsequently loaded onto a Sephacryl S-100 gel filtration column (XK 16/100, GE Healthcare) equilibrated with AcB (0.2 M, pH 5.0). The bound proteins were eluted at a flow rate of 1.5 mL/min and fractions of 1.5 mL were collected, during 150 min, over 1.9 volumes of column. Fractions with

PG activity were pooled, concentrated by lyophilization and kept refrigerated until use.

Procedure II

The CEE (18 mL) was dialyzed overnight at 5°C against buffer AcB (0.2 M, pH 5.0). After dialysis, the solution was freeze-dried, re-suspended in AcB and loaded onto a SP-Sepharose FF cation-exchange column (XK 26/20, GE Healthcare) equilibrated with the same buffer. The bound proteins were then eluted with a linear gradient of NaCl (0–0.5M) over four column volumes at a flow rate of 3.0 mL/min during 150 min. Fractions of 6.0 mL were collected and those with PG activity were pooled, concentrated by lyophilization and kept refrigerated until use.

Enzyme characterization

Molecular weight

The molecular weight of the purified enzyme was determined by the SDS-PAGE, using a low molecular weight marker (14.5 a 94 kDa, GE Healthcare).

Effect of pH on the purified polygalacturonase activity and stability

The effect of pH on the purified PG activity was determined by incubating the reaction mixture at pH values ranging from 2.6 to 6.0 under the standard enzyme assay conditions. The pH stability of the enzyme was evaluated by measuring the residual activity under standard enzyme assay conditions after incubating the pure enzyme without substrate for 24 h at 5°C at various pH from 2.6 to 7.0. The buffer employed in these measurements was citric phosphate buffer (CPB, 0.1M citric acid and 0.2 M Na₂HPO₄). The pH was initially adjusted to pH 5.0 with NaOH and then recalibrated to the desired pH with HCl or NaOH. All the experiments were conducted in triplicate and the results showed the mean values of the activities.

Thermal stability of the purified PG

For the determination of the thermal stability, the enzyme was pre-incubated over a range of temperature from 37°C to 60°C in the CPB at pH optimum. Residual activity was measured during 10 h, under the standard enzyme assay conditions. All the experiments were conducted in triplicate and the results showed the mean values of the activities.

Enzyme kinetics

The Michaelis constant (K_m) and V_{max} values of purified PG were determined from Lineweaver-Burk plots of enzyme activity measured under the standard enzyme assay conditions with polygalacturonic acid (PGA; Sigma) as the substrate at concentrations between 0.05 to 2.5 g/L in BCP at pH optimum.

Influences of metal ions on the purified PG activity

Before the assay, the enzyme solution was dialyzed in order to remove all the interfering substances. The effect of cations such as Cu⁺², Ca⁺², Fe⁺³, K⁺, Mg⁺², Mn⁺² and Zn⁺² on enzyme activity was tested in the reaction medium under the standard enzyme assay conditions.

Analytical techniques

Enzyme activity assays

PG activity was assayed by measuring the reducing groups released after incubation of reaction medium at 30°C for 10 min (pH 5.0) by dinitrosalicylic acid method using PGA as the substrate (Miller 1959). A calibration curve was made with galacturonic acid (GA, Sigma) as standard. One unit of PG was defined as the amount of enzyme which releases 1 µmol of GA per minute.

Protein estimation

Protein concentrations were determined by the Bradford method using bovine serum albumin (Sigma) as standard (Bradford 1976).

Assay of maceration activity

To evaluate the maceration capacity of the purified PG on cassava tissues, cassava was peeled and cut into pieces (3–4 mm on each side), placed in Petri dishes, submerged in a purified PG solution in AcB (0.2 M, pH 5.0) and incubated at 30°C for 2 h. Maceration activity was estimated from the loss of coherence of tissue and by microscopic observations. Blanks were prepared with heat-denatured enzyme.

RESULTS AND DISCUSSION

Enzyme purification

The results of the purification process of PG by Procedure I are summarized in Table 1. Elution profiles of PG on Q Sepharose FF anion-exchange

column and Sephacryl S-100 gel filtration column are shown in Figures 1 and 2, respectively.

Table 1 - Summary of *W. anomalus* PG purification by Procedure I.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)
CEE (10 ×)	731.3	4.93	148	100
Sephadex G-2	488	2.604	188	66
Sepharose Q	324.5	1.26	258	44
Sephacryl S-100	159	ND	ND	21

ND: not detected, CEE: concentrated enzymatic extract.

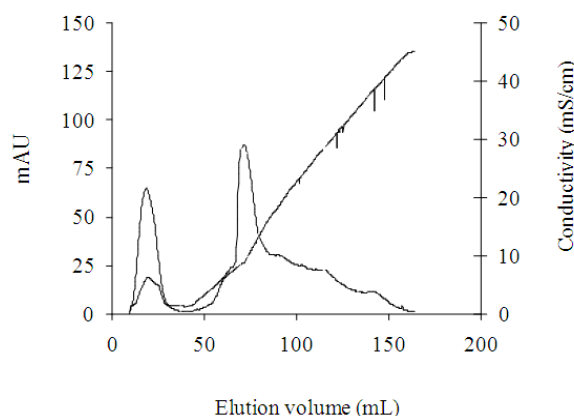


Figure 1 - Elution profile of *W. anomalus* PG on Q Sepharose anion-exchange column (XK 26/20), equilibrated with AcB. Elution of bound proteins with a linear gradient of NaCl (0 - 0.5 M) in AcB, column volume: 7.5, flow rate: 1.5 mL/min, time of elution: 180 min.

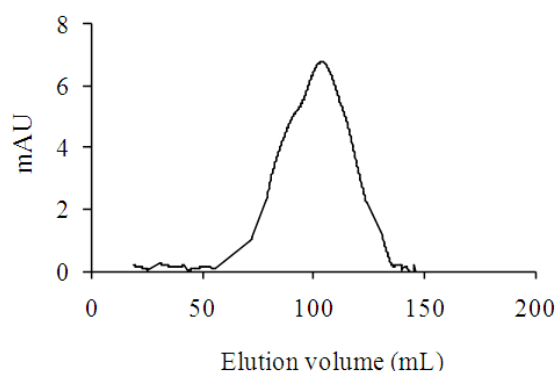


Figure 2 - Elution profile of *W. anomalus* PG on Sephacryl S-100 gel filtration column (XK 16/100), equilibrated with AcB (0.2 M, pH 5.0). Elution of proteins with AcB, column volume: 1.9, flow rate: 1.5 mL/min, time of elution: 150 min.

From Sephadex G-25 column, a fraction 6-13, free of salts, was collected, recovering 66% of initial enzyme activity (Table 1). During anion-exchange chromatography, the protein with PG activity did not bind to Q-Sepharose FF column; others contaminating proteins were eluted at high ionic strength (Fig. 1). In this step, 44% of the initial enzyme activity was recovered (Table 1). After the subsequent purification step using Sephacryl S-100 gel filtration column, PG activity was eluted as a single peak yielding 21% of the original PG activity (Fig. 2). A summary of the purification process of PG by Procedure II is presented in Table 2. Elution profiles of PG on SP-Sepharose FF cation-exchange column is shown in Figure 3. Figure 4 shows the SDS-PAGE of proteins obtained during the different steps of purification.

Table 2 - Summary of *W. anomalus* PG purification process by Procedure II.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)
CEE	1,341	8.12	165	100
Dialysis	903	5.06	179	67
SP-Sepharose	746	3.33	224	56

CEE: concentrated enzymatic extract.

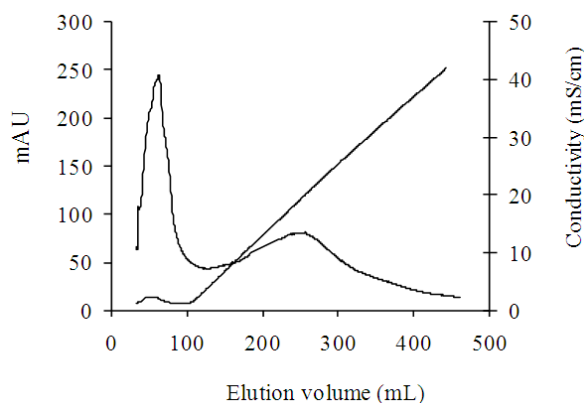


Figure 3 - Elution profile of *W. anomalus* PG on SP-Sepharose cation-exchange column (XK 26/20), equilibrated with AcB (0.2 M, pH 5.0). Elution of proteins with linear gradient of NaCl (0–0.5M) in AcB, column volume: 4, flow rate: 3 mL/min, time of elution: 150 min.

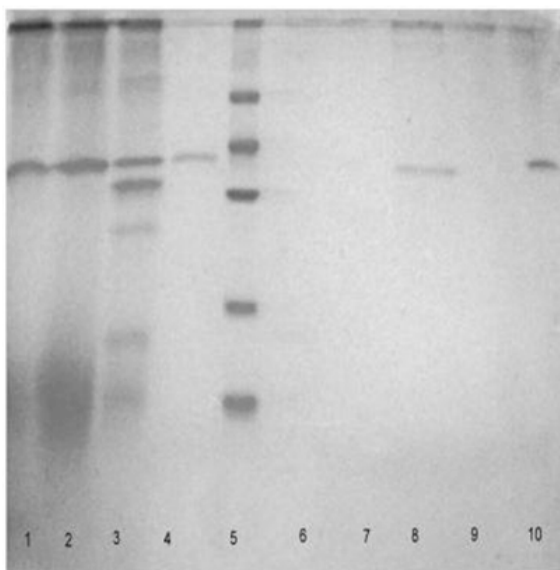


Figure 4 - SDS-PAGE with Coomassie staining of the purification steps of *W. anomalus* PG. Lane 1: concentrated enzymatic extract (CEE). Lane 2: after dialysis. Lane 3: after desalted on a Sephadex G-25 column. Lane 4: after Q Sepharose chromatography. Lane 5: low molecular weight marker. Lane 6: after Q Sepharose chromatography (without PG activity). Lane 7: after Sephacryl S-100 chromatography (fraction 26-40, without PG activity). Lane 8: after Sephacryl S-100 chromatography (fraction 50-70). Lane. 9: after SP-Sepharose chromatography (fraction 7-13), without activity. Lane 10: after SP-Sepharose chromatography (fraction 26-70).

After dialysis, 67% of the initial enzymatic activity was recovered (Table 2). From cation-exchange chromatography a fraction (33-54) with PG activity was obtained, recovering 56% of initial enzyme activity and 1.3-fold increase in PG specific activity (Fig. 3). The protein in lane 4 (after Q Sepharose chromatography), lane 8 (after Sephacryl S-100 chromatography) and lane 10 (after SP-Sepharose chromatography) showed a single band on 10% SDS-PAGE (Fig. 4). PG produced by *S. cerevisiae* CECT1389, grown on YNB medium composed of glucose and PGA, was purified in a single step by size-exclusion chromatography from the culture filtrate (Blanco et al. 1994). Pedrolli et al. (2009) reported that pectic enzymes purifications were performed mainly by the chromatographic techniques. *A. giganteus* PG was purified after two simple steps: protein precipitation and anion-exchange

chromatography. Most of methods for purifying the fungal and bacterial PGases that have been published are associated with considerable enzyme losses, mainly caused by the relatively high number of steps required during the purification process (Pedrolli and Carmona 2010). The purification process described for *W. anomalus* in the present study, using procedure II, could be useful for future industrial scale application. By means of this process, high yields of the enzyme could be obtained in few stages.

Molecular weight

The SDS-PAGE revealed an apparent molecular weight of 43 kDa for *W. anomalus* PG (Fig.4). It has been reported that the molecular weight of PGases from different sources usually ranged between 40 and 60 kDa (Jayani et al. 2005; Pedrolli and Carmona 2010).

Effect of pH on PG activity and stability

The effect of pH on purified PG activity is shown in Figure 5. Figure 6 represents the values of enzymatic residual activity after incubating the enzyme at different pH for 24 h at 5°C.

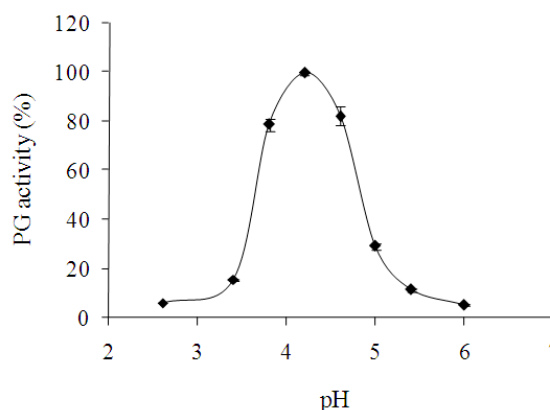


Figure 5 - Effect of pH on purified PG activity produced by *W. anomalus*. PG activity was determined as a percentage of the enzyme activity under standard enzyme conditions.

PG secreted by *W. anomalus* exhibited maximal activity at pH 4.2 (Fig. 5). Figure 6 showed that pure enzyme was stable at a pH range from 3.5 to 6.0, after incubation time of 24 h at 5°C. The analysis of variance revealed no significant differences between these values ($p < 0.05$). The enzyme retained 86% of its activity at pH 7.0. The optimum pH for a purified PG of *S. cerevisiae* CECT1389 was 5.5 and this enzyme was stable in

the pH range of 4.5 to 6.0 (Blanco et al. 1994). Blanco et al. (1999) reported that yeasts PGases exhibited an optimum pH in the acidic region between 3.5 and 5.5. The optimum pH for PG produced by *S. cerevisiae* UCLMS-39 after a purification process was 3.5 (Fernández-González et al. 2004).

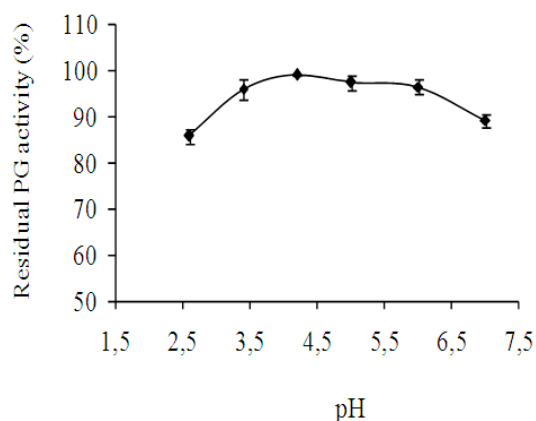


Figure 6 - Effect of pH on purified PG stability produced by *W. anomalus*. PG activity was determined as a percentage of the enzyme activity under standard enzyme conditions.

Thermal stability of PG

The effect of temperature on the purified PG stability produced by *W. anomalus* is shown in Figure 7.

Figure 7 showed that in the absence of substrate, purified PG was stable at 37, 43 and 49°C for 10 h of incubation at optimum pH. The analysis of variance revealed no significant differences between these values ($p < 0.05$). At 55°C, the enzymatic activity decreased and retained only 37% of the initial activity after 15 min of incubation. The thermal stability of PG produced by *W. anomalus* was similar to that reported for PGases from others yeasts such as *S. cerevisiae* IM1-8b and *S. cerevisiae* 1389, which were quite stable in the 20-50°C temperature range but were inactivated (80%) within 5 min at 55°C (Blanco 1997). Two PGases of *Cryptococcus* sp. (named p36 and p40) remained stable up to 40°C for 30 min (Miura et al. 2001). The optimum temperature for PG produced by *S. cerevisiae* UCLMS-39 was 50°C; higher from this temperature, the activity gradually decreased (Fernandez-Gonzalez et al. 2004). The knowledge of enzyme stability is

important to maintain the desired level of enzyme activity over a long period of time and improve its stability for an efficient application in an industrial process (Martins et al. 2007). Besides after any application, the enzyme has to be inactivated, so the knowledge of thermal inactivation has great importance too (Tari et al. 2008).

Some authors reported that during the purification procedure, some protein stabilizing factors might be lost (Naidu and Panda 2003; Martins et al. 2007; Pedrolli and Carmona 2010). Data published previously with crude PG from *W. anomalus* obtained in YNB medium showed that enzyme maintained 78% of its residual activity of after 30 min at 55°C (Martos et al. 2013b). These results suggested that PG lost stability after the purification process.

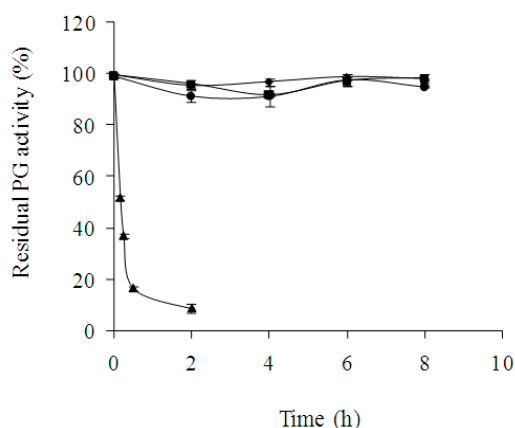


Figure 7 - Effect of temperature on purified PG stability produced by *W. anomalus*. Symbol: ◆37°C, ■ 43°C, ●49°C, ▲ 55°C. PG activity was determined as a percentage of the enzyme activity under standard enzyme conditions.

Kinetic parameters

Kinetic parameters such as V_{max} and K_m were determined from the regression lines of Lineweaver-Burk plots. The V_{max} and K_m values obtained for the PGA were 0.26 mmol/L.min and 0.173 mg/mL ($R^2: 0.901$), respectively. Similar K_M values have been described for several yeast strains, such as 0.57, 0.09, 0.62 and 0.59 mg/mL for PGases produced by *Cryptococcus albidus* (Federichi 1985), *G. lactis* (Blanco et al. 1994), *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* IMI-8b (Gummadi and Panda 2003), respectively.

Effects of various cations on PG activity

The effects of various cations on purified PG activity is shown in Figure 8.

Cu^{+2} , Fe^{+3} , Mg^{+2} , Mn^{+2} and Zn^{+2} did not show any significant effect on the purified PG activity under the assay conditions, whereas the residual PG activity was 67 and 56% in the presence of Ca^{+2} and K^{+} , respectively. It was reported that PGases tended to undergo changes in their physical and chemical properties in the presence of some ions. Therefore, it was necessary to test the effects of a number of representative cations in the reaction medium on PG activity (Miura et al. 2001).

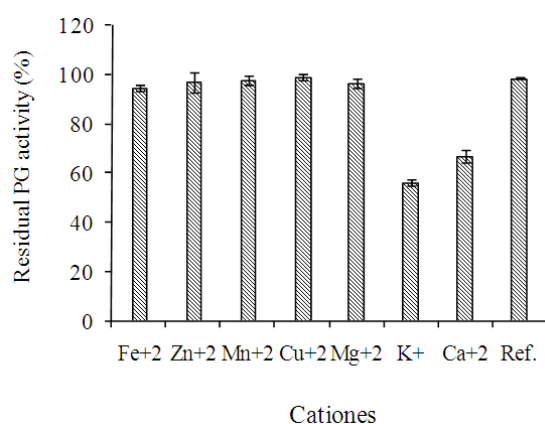


Figure 8 - Effects of cations on purified PG activity produced by *W. anomalus*. PG activity was determined as a percentage of the enzyme activity under standard enzyme conditions.

Assay of maceration activity

Purified PG was able to macerate the cassava tissue as shown by the loss of coherence of the tissue. Microscopic examination of the maceration product showed single cells. These results confirmed the maceration capacity of PG as it was reported previously with the supernatant of *W. anomalus* (Martos et al. 2013a).

CONCLUSIONS

Polygalacturonase from the culture supernatant of *W. anomalus*, grown on YNB medium with glucose and pectin, was efficiently purified using a two-step procedure with a high recovery (dialysis and cation-exchange chromatography). PG exhibited an optimum pH in the acidic region, a high stability over a large pH range and was stable

up to 50°C, suited to most fruit and vegetable processing applications. These properties suggested that this PG could be a potential candidate for different applications in food industry, mainly in the maceration of cassava tissues of regional interest in the province of Misiones, Argentina.

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