



**A CONTRIBUTION TO THE CHARACTERIZATION OF PROTOPECTINASE SE, AN
ENDOPOLY GALACTURONASE WITH PECTIN-RELEASING ACTIVITY FROM
*Geotrichum klebahnii***

**UNA CONTRIBUCIÓN A LA CARACTERIZACIÓN DE PROTOPECTINASA-SE,
UNA ENDO-POLY GALACTURONASA DE *Geotrichum klebahnii* CON ACTIVIDAD
SOLUBILIZADORA DE PECTINA**

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Abstract

Hydrolysis of polygalacturonic acid (1.8 g.L^{-1}) with 1.25 mg.L^{-1} of a commercial preparation (Pectinase SE from Shikibo Ltd., Japan) containing protopectinase SE (PPase-SE) activity for 80 min released a reducing power equivalent to $\approx 400 \text{ mg.L}^{-1}$ of galacturonic acid monohydrate, yielding oligogalacturonates with an average polymerization degree of around 4.

Thermostability of PPase-SE was positively affected by enzyme concentration. Enzyme activity of a solution containing 12.5 mg.L^{-1} of Pectinase SE in 20 mM sodium acetate buffer, pH 5.0, quickly dropped to less than 20 % after 80 s of vortexing. Enzyme pre-incubation (37°C , 30 min) with Ca^{2+} , Mg^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Zn^{2+} and Mn^{2+} (0.1, 1.0 and 10.0 mM) did not affect the activity except in the case of Cu^{2+} (10 mM). Ca^{2+} (2.5 mM) and Mg^{2+} (2.5 mM) in the reaction mixture caused inhibition and activation, respectively. Ca^{2+} inhibition was partially reverted by Mg^{2+} . The enzyme is not inhibited by products. K_m and V_{max} value for PGA was determined to be $0.198 \pm 0.013 \text{ g.L}^{-1}$ and $0.0509 \pm 0.0032 \mu\text{mol.mL}^{-1}.\text{min}^{-1}$ respectively. This K_m value is one of the lowest reported for microbial PGases from different origins.

Keywords: endo-polygalacturonase, kinetic characterization, metal inhibition.

Resumen

La hidrólisis de ácido poligalacturónico (1.8 g.L^{-1} en buffer acetato de sodio pH 5) con una solución 1.25 mg.L^{-1} de un preparado comercial (Pectinase SE, Shikibo Ltd., Japan) de protopectinasa SE (PPasa-SE) por 80 minutos produce un poder reductor equivalente a $\approx 400 \text{ mg.L}^{-1}$ de ácido galacturónico monohidrato, lo que representa un grado de polimerización de aproximadamente 4. La estabilidad térmica de la enzima resulta ser proporcional a la concentración de la misma. Una solución conteniendo 12.5 mg.L^{-1} de pectinasas SE en 20 mM de buffer acetato de sodio pH 5 pierde el 80 % de la actividad al ser agitada en vortex por un período de 80 s a temperatura ambiente. La preincubación de la enzima (37°C , 30 min) con Ca^{2+} , Mg^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Zn^{2+} and Mn^{2+} (0.1, 1.0 and 10.0 mM) no afecta su actividad a excepción del Cu^{2+} (10 mM). El Ca^{2+} (2.5 mM) and Mg^{2+} (2.5 mM) en la mezcla de reacción causan inhibición y activación de la enzima respectivamente. La inhibición causada por el Ca^{2+} es parcialmente revertida por el Mg^{2+} . La enzima no es inhibida por producto. Los valores de K_m y V_{max} para PGA fueron determinados, resultando en $0.198 \pm 0.013 \text{ g.L}^{-1}$ y $0.0509 \pm 0.0032 \mu\text{mol.mL}^{-1}.\text{min}^{-1}$ respectivamente. Este valor de K_m es uno de los más bajos reportados para PGAsas microbianas de diferentes orígenes.

Palabras clave: endo-poligalacturonasa, caracterización cinética, inhibición metálica.

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1 Introduction

Protopectin is the water-insoluble parental substance of pectin found in plant tissues; it yields water-soluble pectin upon restricted depolymerisation. Pectin-releasing or pectin solubilizing enzymes (also called protopectinases, PPases) constitute a heterogeneous group of enzymes able to release soluble pectin from protopectin. The isolation and characterization of several PPases from different microbial origins have been previously reported elsewhere (Sakai, 1992; Sakai *et al.*, 1993). PPases show different catalytic activities. Type-A PPases (with the activities of polygalacturonases, pectate lyases, or pectin lyases) depolymerize the smooth regions whereas type-B PPases (glycan hydrolases) degrade the hairy regions in protopectin releasing in both cases soluble pectin.

Geotrichum klebahnii ATCC 42397 is a yeast-like fungus originally isolated from mandarin peel (Sakai *et al.*, 1978). It produces PPase-SE, an endopolygalacturonase (endo-PGase, EC 3.2.1.15) with pectin releasing activity. The name PPase for this type of enzyme was kept to differentiate it from classical PGases without pectin releasing activity. This enzyme was purified and partially characterized (Rojas *et al.*, 2008; Sakai *et al.*, 1982). The effects of culture conditions and medium composition on PPase-SE production have been previously evaluated and published (Cavalitto *et al.*, 2000; Ferreyra *et al.*, 2002; Cavalitto *et al.*, 2007).

Different potential applications have been proposed for PPase-SE, particularly for the bioprocessing of plant materials. In this sense, PPase-SE proved to be highly efficient for the enzymatic extraction of high-molecular weight pectin from citrus peel (Zapata Zapata *et al.*, 2012), for the maceration of potato tissues to obtain single-cell foods (Nakamura *et al.*, 1995).

Enzyme activity of PPases can be quantified by using either insoluble or soluble substrates. In the former case, activity is evaluated by measuring the solubilization rate of pectin from protopectin (usually prepared in the laboratory from lemon albedo). Nevertheless, this assay, which is time-consuming for routine analysis, is very difficult to standardize due to substrate variability (e.g. type of starting plant tissue, degree of ripeness, preparation procedure, etc.). In addition, it has been shown that particle size of protopectin has a deep effect on enzyme activity (Cavalitto *et al.*, 1997). In order to avoid these disadvantages, an assay method using a soluble substrate (polygalacturonic acid) based on its PGase

activity was proposed for PPase-SE and correlated with its pectin solubilizing activity (Cavalitto *et al.*, 1999).

The aim of this work was to contribute to the characterization of PPase-SE produced by *G. klebahnii*, in particular to study its kinetic properties and also to obtain information on temperature, metal ions and shear effects on enzyme stability and/or activity.

2 Materials and methods

Chemicals : D-galacturonic acid monohydrate (GALA) and polygalacturonic acid (PGA) from oranges were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were commercially available and of analytical grade.

Enzyme source : A commercial enzyme preparation (Pectinase SE from Shikibo Ltd., Japan) containing PPase-SE activity was used. A stock enzyme solution in 20 mM sodium acetate buffer, pH 5.0 (AcB), containing 250 mg.L⁻¹ of the product, was used for the characterization of the PPase-SE kinetic behavior based on its PGase activity.

Enzyme activity : The enzyme activity of PPase-SE was evaluated as PGase according to the method previously proposed (Cavalitto *et al.*, 1999). One unit of PGase activity was defined as the amount of enzyme that releases a reducing power equivalent to 1 μ mol of GALA per minute.

Statistical analysis : All analyses were performed at least in triplicate with the exception of those used for the calculation of K_m and V_{max} , which were performed in quintuplicate. The data were expressed as means \pm standard deviations. Univariate analysis of variance (ANOVA) was employed on the data for PGase activity.

3 Results and discussion

3.1 Characterization of the enzyme activity

Degree of PGA hydrolysis. Time course for the degree for PGA hydrolysis by PPase-SE was determined. For this purpose, a series of reaction mixtures (composed of 4.5 ml of a 2.0 g.L⁻¹ PGA solution in AcB and

0.5 mL of an enzyme solution diluted 1:20 in AcB)

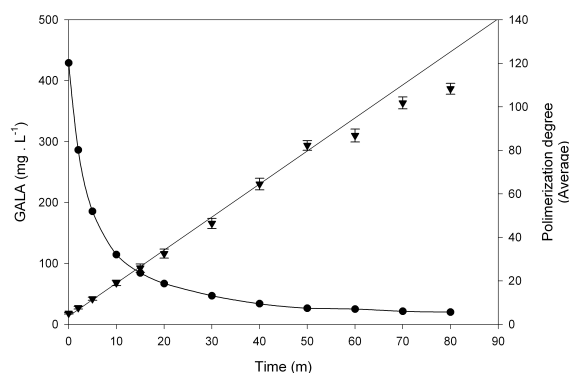


Fig.1. Relationship between the released reducing power, expressed as equivalents of GALA (▼), and the average degree of polymerization (●) at different incubation times.

were incubated at 37°C at different reaction times up to 80 min. After incubation, the reaction mixtures were heated in a boiling water bath for 3 min to inactivate the enzyme. These solutions were called final product solutions (FPSs) and their contents in equivalent amounts of reducing groups released during the incubation were calculated (Fig. 1). The initial polymerization degree of PGA (in average), calculated by comparing the amount of PGA (in mass) dissolved in the substrate solution and the equivalent reducing power (as AGA) of the reaction blanks, was estimated to be around 110 ($M_r \approx 21,000$). This value is in good agreement with the information provided by the PGA supplier (personal communication).

The degree of hydrolysis reached in FPS at the end of incubation time (80 min) yielded amount of reducing groups equivalent to around 400 mg·L⁻¹ of GALA. Considering this value of reducing power, the average polymerization degree of the final reaction products (oligogalacturonates) was estimated to be around 4. Samples that were incubated at longer reaction times than 80 min gave the same result (data not shown). This value can be considered as the enzymatic hydrolysis limit for PPase-SE. As can be seen in Fig 1, the reaction rate begins to decrease after 60 minutes of reaction. This behavior of the reaction speed may be due to a large degradation of the substrate or to a product inhibition effect. In order to clarify the real cause of that, different amounts of FPS were added to the reaction mixture and initial speed was determinate in every case.

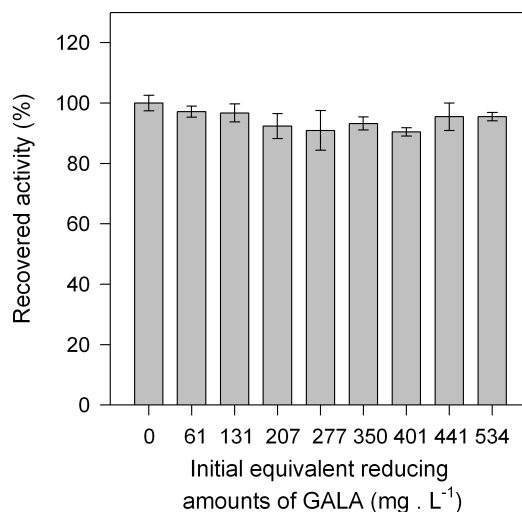


Fig. 2. Effect of the addition of different amounts of reaction products (expressed as initial equivalent reducing amounts of GALA) to the reaction mixture on PGase activity of PPase-SE. Enzyme stock solution was diluted 1:20. Reaction time was 10 min. Values are means \pm SD of four replicates. See text for experimental details.

As can be seen in figure 2, there is not an inhibition effect caused by the final products of the reaction. Because of that, it can be concluded that polymerization degree of the oligogalacturonic acid is the cause of the decrease in the reaction rate. This result is in good agreement with the mechanism proposed for the pectin solubilization by PPase-SE. This enzyme reacts with the pectin molecule in protopectin at sites with at least four nonmethoxylated galacturonic acid chains and cleaves their glycosidic linkages (Sakai *et al.*, 1993) so that, oligogalacturonates with less than five AGA residues are not good substrates for the enzyme. However, other reports have found that different endo-PGases of microbial origin can yield galacturonate dimers and in some cases trimers as final reaction products of PGA hydrolysis (Rombouts *et al.*, 1980).

Effect of temperature on enzyme stability. Stock solutions (undiluted and 1:20 diluted in AcB) were incubated at 0 and 37°C. At regular time intervals samples were withdrawn and immediately analyzed for residual PGase activity (Fig. 3). As can be seen, PPase-SE is more stable at the higher concentration either at 0 or 37°C. After 4 h of incubation at 37°C the undiluted sample retained 80 % of the initial activity

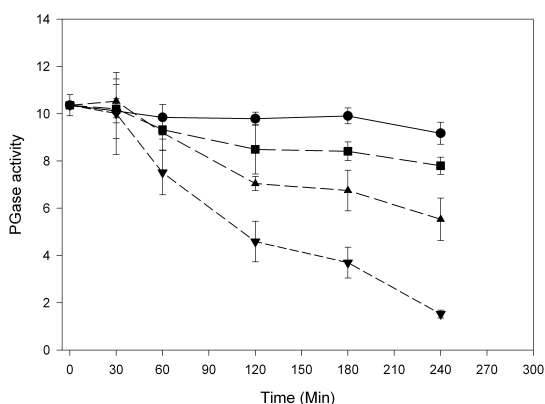


Fig. 3. Activity of PGase after incubation at 0 and 37°C. Undiluted sample (0°C ●, 37°C ■); 1:20 diluted sample (0°C ▲, 37°C ▼).

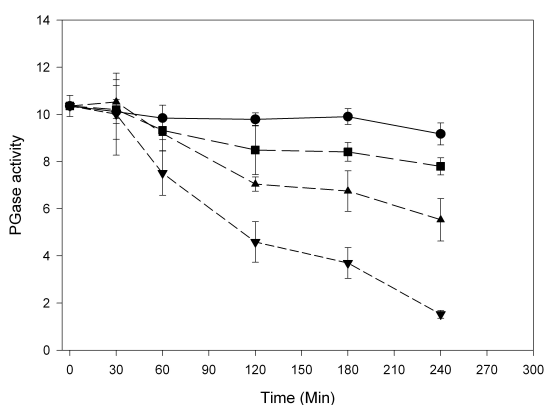


Fig. 4. Effect of stirring on PPase-SE activity.

whereas this value fell down to less than 20% for the diluted one. Similar behavior has been found for the PGases produced by *Verticillium albo-atrum* (Wang *et al.*, 1970) and *S. cerevisiae* (Blanco *et al.*, 1994).

Effect of shear on enzyme activity. Due to the stability of the enzyme decrease with the decrease of the concentration and any inconsistencies found in the routine measurement of activity (after corresponding dilution of the samples) we attempted to study the shear effect on PPase-SE activity. For this purpose, 2 mL of the enzyme stock solution was cooled, diluted 1:20 with AcB in a test tube and kept in ice-water bath. This solution was stirred in vortex and samples of 10 μ L were withdrawn every ten seconds. Fig. 4 shows the recovered enzyme activity. It can be viewed that the enzyme activity quickly drops to less than 20 %

after 80 s of stirring. Colombie *et al.* (2000) found a similar effect of stirring on lysozyme. It is usually believed that shear forces are the responsible of protein damage; however, the emerging result of most studies is that shear in the fluid mechanical sense is unlikely by itself to damage most proteins in solution and that interfacial phenomena are critically important. In particular, moving gas-liquid interfaces can be very deleterious (Thomas *et al.*, 2011). Probably, this could be the case when a solution containing PPase-SE is vortexed under the experimental conditions above mentioned.

The potential instability of PPase-SE in stirred solutions should be taken into account for the design of the different steps involved in its production and use where shear forces are necessary implicated.

Effect of some metal ions on enzyme stability and activity. The effect of some cations commonly used as supplements in culture media was investigated. Cations tested were Ca^{2+} , Mg^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Zn^{2+} , and Mn^{2+} at three different concentration levels (0.1, 1.0 and 10 mM). The enzyme stock solution was incubated under the presence of these cations at 37°C for 30 min. Then, the cation-enzyme solutions were diluted 1:20 in AcB and their enzyme activities were immediately assayed (thus, final cation concentrations in the corresponding reaction mixtures were 5×10^{-4} , 5×10^{-3} and 5×10^{-2} mM, respectively). No significant differences ($p < 0.05$) were observed in all measured enzyme activities in comparison with non-cation treated enzyme samples except when 10 mM Cu^{2+} was used during the incubation. In this case, the recovered activity was 75 %. It is interesting to mention that Wang and Keen (1970) reported a value of $K_i = 7.0 \times 10^{-3}$ mM for an endo-PGase of *Verticillium albo-atrum*, which represents 58 % inhibition for a Cu^{2+} concentration of 5×10^{-2} mM. Therefore, direct measurement of PPase-SE activity can be carried out in almost all-crude culture samples without any previous treatment to eliminate residual cations.

As previously mentioned, PPase-SE has a great potential for the enzymatic pectin extraction from citrus peels. This particular raw material contains significant amounts of Ca^{2+} as an inorganic component in the protopectin structure (Fry, 1988); it could be solubilized during pectin extraction. It has been reported that Ca^{2+} , which interacts with polygalacturonans to form aggregates, is inhibitory at concentrations as low as 0.4 mM. The aggregates thus formed are considered to be unavailable for enzymatic attack, and they may actually bind to and

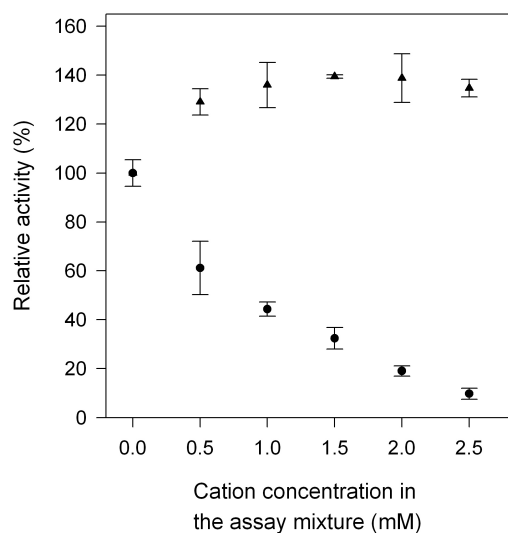


Fig. 5. Effect of different concentrations of Ca^{2+} (●) and Mg^{2+} (▲) in the reaction mixture on PGase activity of PPase-SE. Enzyme stock solution was diluted 1:20. Reaction time was 10 min. Values are means \pm SD of three replicates.

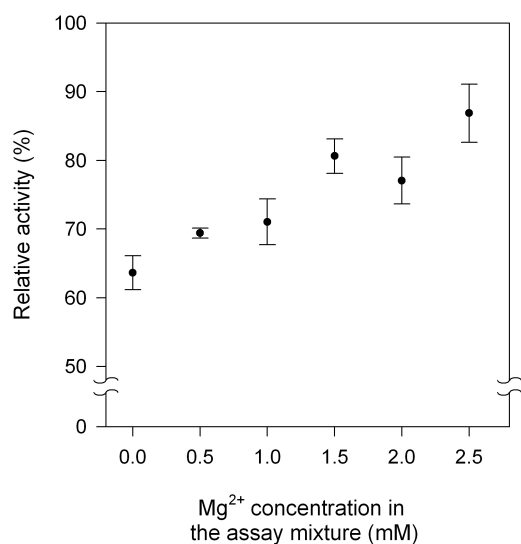


Fig. 6. Partial reversion of Ca^{2+} (1.0 mM) inhibition on PGase activity of PPase-SE by different concentrations of Mg^{2+} in the reaction mixture. Enzyme stock solution was diluted 1:20. Reaction time was 10 min. Values are means \pm SD of three replicates.

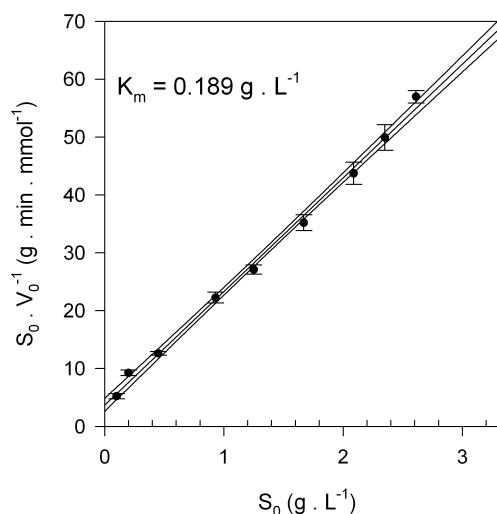


Fig. 7. Hanes-Hulting plot for PGase activity of PPase-SE (99% Confidence interval). Enzyme stock solution was diluted 1:20. Reaction time was 10 min. Values are means \pm SD of five replicates. S_0 = Initial PGA concentration ($\text{g}\cdot\text{L}^{-1}$). V_0 = Initial reaction rate ($\text{mmol}\cdot\text{L}^{-1}\cdot\text{min}^{-1}$).

immobilize the enzyme (Burns, 1991). Bearing this in mind, we studied the effect of Ca^{2+} , at higher concentrations than those above tested, in the reaction mixture with no previous incubation of the metal with the enzyme. For this purpose, the reaction mixture was supplemented with increasing amounts of Ca^{2+} (from 0 to 2.5 mM). Enzyme activity detected was strongly inhibited (Fig. 5). Mg^{2+} was also tested with the same procedure and showed an activating effect on enzyme activity (Fig. 5). No differences in the above results were observed with both cations when used as either chlorine or sulphate salts (Data not shown). Moreover, the inhibitory effect of Ca^{2+} at a concentration of 1.0 mM was partially reverted by Mg^{2+} (Fig. 6). The exact nature of this behavior is out of the scope of this research and it remains to be elucidated.

Determination of kinetic constants. Initial rate of PGA hydrolysis was determined for substrate concentrations ranging from 0.1 to 2.5 $\text{g}\cdot\text{L}^{-1}$. Fig. 7 shows the Hanes-Hulting plot that corresponds to the Michaelis-Menten equation rearranged according to the following equation (Kubi, 1991).

$$\frac{S_0}{V_0} = \frac{K_m}{V_{max}} + \frac{S_0}{V_{max}} \quad (1)$$

As can be seen, initial PGA hydrolysis is appropriately

described by a Michaelis-Menten kinetics. The K_m and V_{max} for PGA, as calculated from a linear regression analysis of the equation, were $0.189 \pm 0.013 \mu\text{g.mL}^{-1}$ and $0.0509 \pm 0.0032 \mu\text{mol.mL}^{-1}.\text{min}^{-1}$ respectively. This K_m value is one of the lowest reported for microbial PGases from different origins (Whitaker, 1990).

Conclusions

According to the properties described in this work, mainly the resistant to metal and inhibition by products inhibition, PPase SE results to be a good alternative to use in vegetable tissue maceration (for baby food preparation), enzymatic pectin extraction and fruit juice clarification.

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