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Quantification of protopectinase SE, an endopolygalacturonase with pectin-releasing activity from *Geotrichum klebahnii*

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Abstract

Pectin releasing activity of protopectinase SE (PPase-SE) from *Geotrichum klebahnii* (= *G. penicillatum* = *Trichosporon penicillatum*) ATCC 42397 was determined using different batches of lemon protopectin as substrate. Results obtained showed a high degree of variability depending on the batch of protopectin used. As PPase-SE also shows polygalacturonase (PGase) activity, a method for the assay of this activity was optimised. The best assay conditions were: substrate (polygalacturonic acid) concentration of 2.0 g l^{-1}, reaction time of 10 min and up to 0.17 PGase units per test tube.

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. Several PPases from different microbial origins were isolated and characterised (Sakai 1992, Sakai *et al*. 1993). PPases show different catalytic activities and they have been classified according to their reaction sites in the protopectin molecule. Type-A PPases (with the activities of polygalacturonases, pectate lyases, or pectin lyases) depolymerise the smooth regions whereas type-B PPases (glycan hydrolases) degrade the hairy regions in protopectin releasing in both cases soluble pectin.

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 from lemon). Recently, Cavalitto *et al.* (1997) reported the influence of enzyme quantity, reaction time, and particle size of protopectin in this assay. Nevertheless, these authors pointed out that this methodology, which is time-consuming for routine analysis, is very difficult to standardise due to substrate variability (e.g., type of starting plant tissue, degree of ripeness, preparation procedure, etc.).

PPase-SE, a type-A PPase, is produced by *Geotrichum klebahnii* (= *G. penicillatum*) ATCC 42397, a yeast-like fungus originally isolated and named as *Trichosporon penicillatum* SNO-3 by Sakai & Okushima (1978). This enzyme, which releases high molecular weight soluble pectin from citrus peel (Sakai & Okushima 1980), was purified, partially characterised and identified as an endopolygalacturonase (endo-PGase, EC 3.2.1.15) (Sakai & Okushima 1982; Sakai *et al.* 1982). Therefore, its activity can alternatively be assayed with a soluble substrate (polygalacturonic acid) by measuring the releasing rate of reducing groups. A number of methods are available for this purpose (Rexová-Benková & Markovic 1976), but the colorimetric test of Somogyi-Nelson (Somogyi 1952) is preferred.

In the first part of this paper, we show the results obtained when different batches of lemon protopectin were used for the quantification of the pectin releasing activity of PPase-SE. The optimisation of the assay for

the quantification of the PGase activity of PPase-SE is later reported and discussed.

Materials and methods

Chemicals

D-Galacturonic acid monohydrate (GALA) and polygalacturonic acid (PGA) from oranges were from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of analytical grade.

Enzyme sources

A partially purified enzyme preparation (powder form) kindly provided by Prof. T. Sakai (University of Osaka Prefecture, Japan) was used. This preparation shows PPase-SE activity with almost no contamination by other enzyme activities (T. Sakai, pers. commun.). A stock enzyme solution in 20 mM sodium acetate buffer, pH 5.0 (AcB), containing 250 mg l^{-1} of the powder, was used for the optimisation of the PGase activity assay methodology.

A crude final culture supernatant of *G. klebahnii* ATCC 42397 was utilised to quantify its PPase activity, and also to evaluate the proposed method for PGase activity determination of PPase-SE. Up to present no other extracellular enzyme except PPase-SE has been reported for this strain. A glucose-based medium was used for cultivation of the microorganism. Details on culture conditions and factors affecting both, the behaviour of this strain and PPase-SE production, will be published elsewhere.

Procedure for protopectin preparation

Different batches of protopectin were prepared as previously reported (Cavalitto *et al*. 1997). Commercial lemons (*Citrus limon* Burm, cv. Lisbon), cultivated in the San Pedro region (Buenos Aires Province, Argentina), harvested during the winter of 1995, 1996, 1997 and 1998 (at the same apparent degree of ripeness) were used. The following procedure was carried out to obtain five different batches of protopectin, two of them belonging to lemons simultaneously harvested in 1997. The albedo layer was scooped and immediately mixed with ice-cooled 96% (v/v) ethanol (1 ml g^{-1} wet albedo) to inactivate native enzymes, particularly pectinesterase (PE, EC 3.1.1.11) (Fry 1988). This suspension was ground in a food processor and kept at 4 °C for 24 h. Particles were filtered through a nylon voile and washed several times with cold distilled water until the soluble substances that react with the carbazole-sulfuric acid reagent (McComb & McCready 1952) were washed off yielding five different soluble pectin free suspensions of protopectin. These suspensions were sifted through meshes 16 and 20. Particles that passed mesh 16 and retained in mesh 20 were washed with acetone and then dried at 30 °C. The five different dry materials obtained were sifted and the fractions that passed mesh 50 and retained in mesh 60 were kept at 4 °C until used as enzyme substrates.

Enzyme assays

PPase activity of PPase-SE using different batches of protopectin as substrate was evaluated as previously described (Cavalitto *et al*. 1997). One unit of PPase activity was defined as the activity that releases soluble pectic substances corresponding to the reducing power of 1 μmol of GALA per minute.

PGase activity of PPase-SE was assayed by measuring the releasing rate of reducing groups from PGA by the method of Somogyi-Nelson with GALA as standard. The substrate solution (450 μl) in AcB was loaded in triplicate test tubes. Fifty microliters of enzyme solution (diluted in the same buffer) was added and the tubes were incubated at 37 °C. Reaction was stopped by the addition of 500 μl of Somogyi reagent, and the tubes were transferred to a boiling water bath for 10 min. Tubes were cooled in an ice-water bath for 10 min, the content mixed with 500 μl of Nelson reagent and kept at room temperature for 30 min. The resulting solution was diluted with 4.5 ml water and the absorbance at 660 nm was measured. A standard curve up to 300 mg l^{-1} of GALA was used. Samples containing higher amounts of reducing groups were conveniently diluted before the addition of the Somogyi reagent. One unit of PGase activity was defined as the activity that releases a reducing power equivalent to 1 μmol of GALA per minute.

As the above-mentioned procedure is time-consuming, a time-saving method was devised to simultaneously quantify a large number of samples. For this purpose, the enzyme solutions to be tested were cooled and mixed with the cold substrate solution contained in Pyrex test tubes (i.d. 13 mm, 110 mm length, 0.85 mm wall thickness, previously cooled in an ice-water bath for 10 min) in a wire rack. The rack with the tubes was then transferred to a circulating water bath at 37 °C (water level was around 5 cm above

Table 1. Determination of PPase activity in a culture supernatant of *G. klebahnii* ATCC 42397 using different batches of protopectin. See text for details.

Batch number of lemon prospection	Lemon harvesting (year)	Measured PPase activity (units ml^{-1})
1	1995	0.75 ± 0.05
2	1996	0.68 ± 0.09
3	1997	0.49 ± 0.04
4	1997	1.44 ± 0.08
5	1998	1.42 ± 0.10

Values are means ± SD of three replicates.

the bottom of test tubes) for incubation. The reaction was stopped by cooling the tubes in an ice-water bath for 10 min followed by the addition of the Somogyi reagent. Further steps were as above described.

Results and discussion

Determination of PPase activity of PPase-SE

PPase activity of PPase-SE was determined in a culture supernatant of *Geotrichum klebahnii* ATCC 42397 using different batches of lemon protopectin. Results are shown in Table 1. As can be observed, values of PPase activity obtained for the same enzyme sample showed great variability even for the two protopectins prepared from lemons harvested in the same season (batches No. 3 and No. 4). Moreover, determinations of PPase activity using batch No. 1 for 3 years gave reproducible results (the enzyme preparation showed high stability while kept in dry condition at 4 °C). The same fact was also found with the other protopectin batches but for shorter periods (2 years for the batch No. 2, etc.). This proved that protopectin is a stable substrate while kept dry in a closed container in refrigerator.

It should be pointed out that the lemons used for protopectin preparation were from the same geographical region and had the same time of harvesting, and the procedure for preparation of the different batches of protopectin was identical in all cases. Variations found in the PPase activity could tentatively be ascribed to possible changes in the chemical composition of lemon cell walls (perhaps in the methoxylation degree), to modifications in their permeability, which could interfere in the accessibility of the enzyme for the reaction site, and/or changes in the adsorption process of the enzyme onto the solid substrate. The origin of these possible changes remains unknown. Studies on these possibilities are in progress and will be reported elsewhere.

Determination of PGase activity of PPase-SE

Since the quantification of PPase-SE activity using its pectin releasing capacity was highly influenced by the protopectin used, it was decided to standardise the assay for its PGase activity.

Substrate concentration. A PGA concentration of 2.0 g l^{-1} was tentatively chosen for the substrate solution. A higher amount of substrate (5.0 g l^{-1}) was also used. In this case, a precipitate of residual PGA, which was formed after the addition of the Nelson reagent to the reaction mixture (Collmer *et al*. 1988), was separated by centrifugation (200 × *g*, 3 min).

Effect of reaction time. Enzyme reaction was carried out at different incubation times with a PGA concentration of 2.0 g l^{-1} and a (tentatively chosen) 1:40 dilution of the stock enzyme solution in AcB (Figure 1).

Linearity of the enzyme assay. Different amounts of enzyme in the reaction tube were used in order to determine the limit of linearity for the assay method (Figure 2). Equivalent reducing amounts of GALA released from PGA (2.0 g l^{-1}) remained proportional to the quantity of enzyme assayed up to 120 μl of stock enzyme solution (diluted 1:20 in AcB) in the reaction test tube; it levelled off at about 500 mg l^{-1} when higher amounts of enzyme were used.

A substantially increased concentration of PGA (5.0 g l^{-1}) in the reaction mixture was also tested. In this case, the method linearity remained constant up to at least of 0.22 PGase units in the test tube, an enzyme activity which releases an equivalent reducing amount of GALA of around 600 mg l^{-1} (Figure 2). Nevertheless, and as previously pointed out, the use of PGA concentrations higher than 2.0 g l^{-1} is not convenient for routine enzyme quantification because it yields a precipitate of residual PGA after the addition of the Nelson reagent which has to be removed by centrifugation.

Proposed technique for PGase activity measurement. According to the above results, we propose to utilise a PGA concentration of 2.0 g l^{-1} for the substrate so-

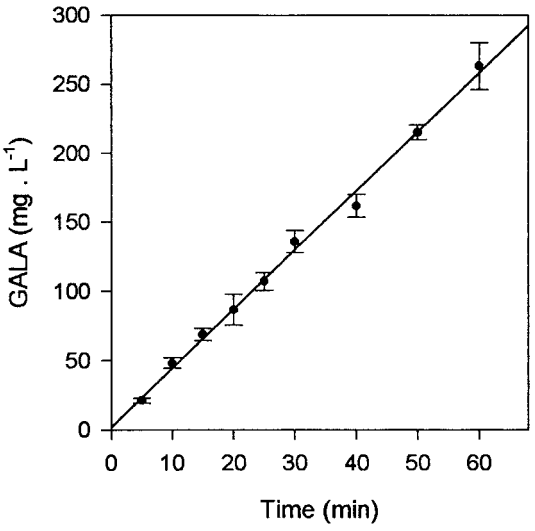

Fig. 1. Time-course of PGA (2.0 g l^{-1}) hydrolysis (expressed as equivalent reducing amount of GALA released from PGA) by PPase-SE (50 μl stock enzyme solution, diluted 1:40). Values are means ± SD of three replicates.

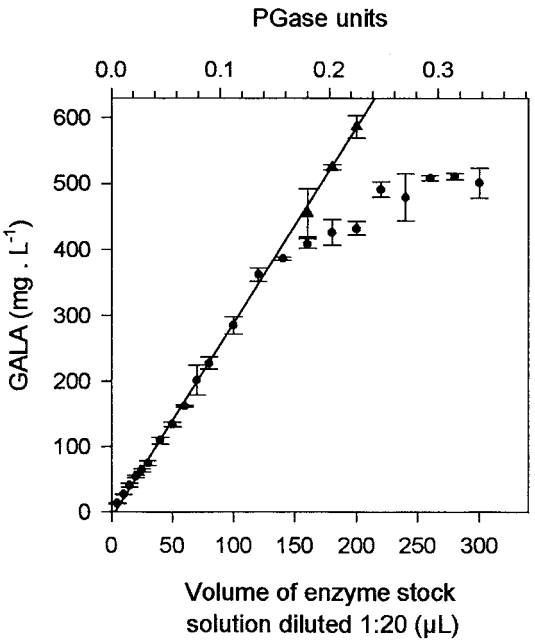

Fig. 2. Relationship between enzyme activity (expressed as equivalent reducing amounts of GALA released from PGA) and amount of PPase-SE (as volume of stock enzyme solution diluted 1:20 in AcB, and as PGase units in the reaction mixture) for two different substrate (PGA) concentrations: (●) 2.0 g l^{-1}, (▲) 5.0 g l^{-1}. Reaction time was 10 min. Values are means ± SD of three replicates.

Table 2. PGase activity (units ml^{-1}) determined by using the method proposed, in different dilutions of a culture supernatant of *G. klebahnii* ATCC 42397. See text for details.

Supernatant dilution	Supernatant PGase activity (units ml^{-1})
1:15	14.3 ± 0.1
1:40	15.0 ± 0.4
1:100*	14.1 ± 0.7

*Reaction time was 30 min.
Values are means ± SD of three replicates.

lution and a reaction time of 10 min for the PGase activity evaluation of PPase-SE. Therefore, in this case, the maximal amount of enzyme activity in the test tube should not exceed 0.12 PGase units (Figure 2). It should be remarked that these reaction conditions could not necessarily be the best ones for the assay of other microbial PGases, or employing a PGA with different chemical characteristics.

Quantification of PGase activity in culture samples

Different dilutions of a crude final culture supernatant of *G. klebahnii* ATCC 42397 (with an almost negligible glucose concentration) were used in order to test if the above-described method for PGase activity quantification of PPase-SE was appropriate for practical purposes. Results are shown in Table 2. As it can be seen, no significant differences were observed among the tested dilutions. In addition, when low enzyme activities have to be determined (as in the case of 1:100 dilution) longer incubation times can be used with no significant differences in the results obtained. This last procedure can also applied for the quantification of PPase-SE activity in culture samples containing interfering residual amounts of reducing sugars. In these cases, an appropriate relationship between sample dilution and incubation time has to be used in order to minimise the absorvance values of the reaction blanks.

Correlation between PPase and PGase activities of PPase-SE

A correlation between PGase (determined with the above-mentioned method) and PPase (measured according to a previously reported technique, Cavalitto *et al.* (1997), using the batch No. 5 of protopectin) activities of PPase-SE was established using different amounts of the stock enzyme solution (Figure 3).

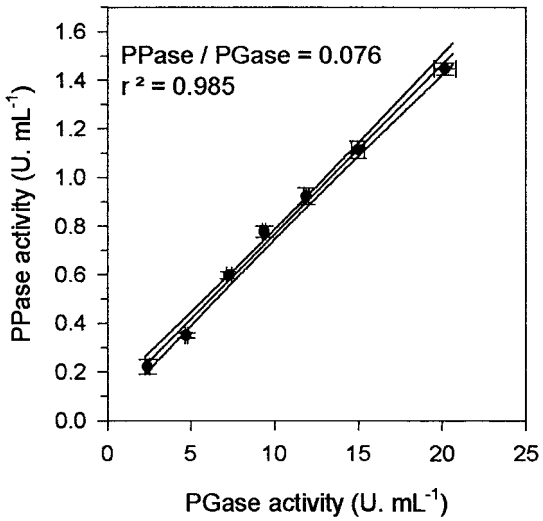

Fig. 3. Relationship between PPase and PGase activities of PPase-SE (95% confidence interval) using different amounts of stock enzyme solution. Values are means ± SD of three replicates.

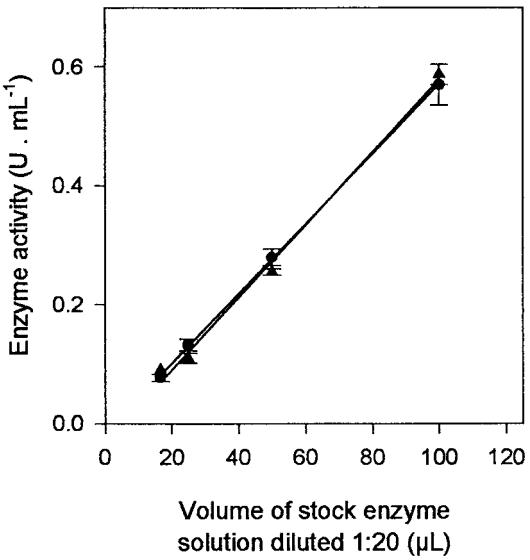

Fig. 4. Comparison between the normal (●) and the time-saving (▲) procedures for the quantification of PGase activity of PPase-SE using different volumes of stock enzyme solution (diluted 1:20). Reaction time was 10 min. Values are means ± SD of three replicates.

Then, the ratio between both activities (PPase/PGase) remained constant with a value of 0.076. The same ratio was calculated from determinations of PPase and PGase enzyme activities in the culture supernatant of *G. klebahnii*. Therefore, PPase activity can be indirectly estimated from data of PGase activity. Nevertheless, PPase values obtained in this way should be observed because they would correspond to the particular batch of protopectin used for PPase quantification.

Time-saving method for PGase activity quantification

When a large number of samples must be analysed, the above-described procedure is tedious and time-consuming as already mentioned. A time-saving method was optimised and used to evaluate different amounts (from 20 to 100 μl) of the diluted (1:20) stock enzyme solution in the reaction test tube and compared with the values obtained with the normal assay procedure (Figure 4). Results obtained with this time-saving method were similar to those achieved using the normal procedure in the range of activities tested.

Conclusions

According to the above results, comparison of pectin releasing activities of PPases among different laboratories, and even at the same laboratory using different batches of protopectin, becomes troublesome. Therefore, quantification of a PPase using its activity on a soluble substrate, which should be known in advance, seems to be advisable. Moreover, the time-saving method for PGase activity determination proved to be a convenient technique when a large number of samples must be simultaneously and routinely tested.

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