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Quantification of pectin-releasing activity of protopectinase-SE from Geotrichum klebahnii

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A method for quantification of a pectin releasing enzyme (PPase-SE) from Geotrichum klebahnii (= Geotrichum penicillatum = Trichosporon penicillatum) ATCC 42397 is reported. PPase activity was determined by measuring the amount of soluble pectin released from lemon protopectin. Particle size of the substrate, reaction time and linearity range of the assay, were analysed. The best assay conditions were a reaction time of 30 min, 20 mg substrate (mesh 60) and up to 0.045 units PPase activity per test tube.

Introduction

Protopectin is the water-insoluble parental substance of pectin found in plant tissues; it yields water-soluble pectin upon restricted depolymerization. Pectin-releasing or pectin-solubilizing enzymes (also called protopectinases, PPases) are a heterogeneous group of enzymes able to release soluble pectin from protopectin. Several PPases from different bacterial or yeast origins were isolated and characterised (Sakai, 1992; Sakai *et al.*, 1993).

PPases have one common characteristic: they are much more active against insoluble forms of specific substrates than against soluble forms. A-Type PPases (with the activities of polygalacturonase, pectate lyase, or pectin lyase) release soluble pectin by restricted depolymerization of the smooth regions in protopectin. Owing to such characteristic, these enzymes release soluble pectin without degrading solubilized pectin at the same time.

Enzyme activity of PPases can be quantified either by using insoluble (protopectin) or soluble substrates. In the former case, which is particularly useful for the determination of the pectin-releasing capacity of PPases, enzyme activity is evaluated by measuring the amount of pectin solubilized from protopectin (usually from lemon). Solubilized pectin is quantified by the carbazole-sulfuric acid method (McComb and McCready, 1952) and expressed as the equivalent reducing amount of galacturonic acid released. Nevertheless, the influence of different factors such as enzyme quantity, reaction time, and particle size of the solid substrate (characteristic of this type of enzyme reactions in heterogeneous systems), has not been reported in detail so far.

Materials and methods

Chemicals

D-Galacturonic acid monohydrate was from Sigma. All other chemicals were of analytical grade.

Enzyme sources

A commercial enzyme preparation (Pectinase SE from Shikibo Ltd., Japan) containing PPase-SE activity was used. This enzyme, which is able to release high molecular weight pectin from citrus peels (Sakai and Okushima, 1980), also shows endo-polygalacturonase activity (endo-PGase, EC 3.2.1.15). A stock enzyme solution in sodium acetate buffer 20 mM, pH 5.0 (AcB), containing 250 $mg.L^{-1}$ of the product, was used for the optimisation of the enzyme assay methodology.

The supernatant of a culture of *Geotrichum klebahnii* (= *Geotrichum penicillatum*) ATCC 42397 was also used to test the technique later described. Details on culture conditions and factors affecting the behaviour of this microorganism and PPase production will be published elsewhere.

Substrate preparation

Protopectin was prepared from commercial lemons (*Citrus limon* Burm). The albedo layer was scooped and mixed with ice-cooled 96% (v/v) ethanol (1 $mL.g^{-1}$ wet albedo) to inactivate native enzymes (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 water-soluble substances that react with the carbazole-sulfuric acid reagent were washed off yielding a soluble pectin free suspension of protopectin. The suspension was sifted through meshes 10, 16, and 20. Particles that passed through mesh 20 were discharged because they had become too small (mesh higher than 120) after drying to be used as the enzyme substrate. Fractions retained in meshes 10 and 16 were suspended in cold water, ground and sifted again. These steps were repeated several times in order to increase the yield of the fraction corresponding to the particles that passed through mesh 16 and were retained in mesh 20. All portions of the material belonging to this fraction were filtered through a nylon voile and, while still wet, were washed with acetone and then dried at 30°C. The dry material obtained was sifted (meshes 35, 40, 50, 60, 80, 100, and 120) and the fractions kept at 4°C until used.

Enzyme assays

Triplicate test tubes, containing 20 mg protopectin and 950 μL AcB, were kept in a water-ice bath. Fifty microliters (unless otherwise stated) of enzyme stock solution was added and the tubes were incubated in a water bath orbital shaker at 37°C. Particle size of protopectin, amount of stock enzyme solution and reaction time varied according to the purpose of the experiment. Reaction was stopped by cooling the test tubes in a water-ice bath. Appropriate control blanks were also run. After the reaction, the mixture was filtered through a conic filter paper (Whatman N0.1). Three millilitres of chilled 32 N H_2SO_4 was added to the test tubes containing 250 μL of filtrate (appropriately diluted in the same buffer), and this was followed by addition of 250 μL of 2 $g.L^{-1}$ carbazole solution in absolute ethanol (this step was carried out in a water-ice bath). The resulting mixture was heated in a boiling water bath for 20 min and allowed to stand at room temperature for 20 to 40 min. Absorbance at 525 nm was measured against a reaction blank. The pectin released was expressed as the equivalent reducing amount of D-galacturonic acid monohydrate (GALA) from a standard curve up to 500 $mg.mL^{-1}$.

Results and discussion

Effect of protopectin particle size

Enzymatic solubilization of pectin from protopectin is an example of heterogeneous catalysis where the surface area of the solid substrate available for the enzyme has a deep influence on the reaction (McLaren and Packer, 1970). Assuming that protopectin particles have a spherical shape, we could estimate the external surface area/unit of volume ratio (A/V) for each fraction using the maximum diameter of the particles that will allow them to pass through the corresponding mesh (Goldberger and Robbins, 1984). Therefore, estimated A/V values, ranging from 10 to 40 mm^{-1} for the tested meshes, were the minimum ones for each case. Pectin released, expressed as GALA, is shown in Fig. 1.

Under these reaction conditions, the equivalent amount of GALA released increased until mesh 50 protopectin was utilised as substrate, and then it levelled off. Therefore, the curve can be divided into two regions. The first one corresponds to those protopectin fractions which have a total substrate surface area insufficient to saturate the amount of enzyme used (available surface area limiting reaction rate). The second one includes those protopectins with comparative higher A/V values (enzyme limiting reaction rate). This behaviour could be explained by assuming that the reaction rate is proportional to the amount of adsorbed enzyme, and that the absorption phenomenon follows the Langmuir isotherm absorption model (McLaren and Packer, 1970). It is interesting to note that in the first region of the curve, the reaction rate is proportional to the external surface area per unit of volume. This fact suggests that, in this case, only the external surface of the substrate is available for the enzyme.

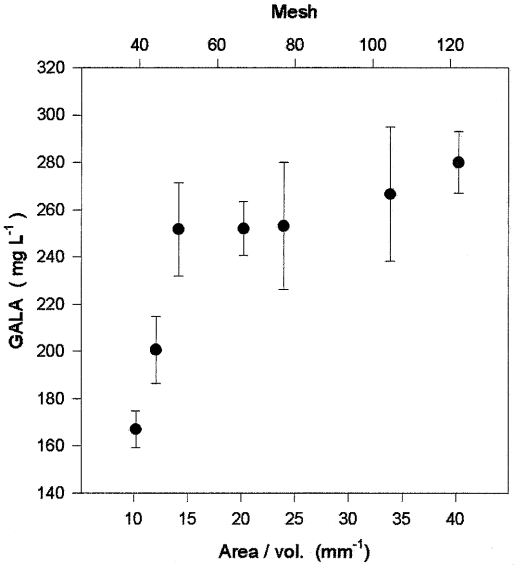

Figure 1 Effect of particle size of the substrate (20 mg) on the GALA released from protopectins of different meshes by PPase-SE (50 μL enzyme stock solution). Reaction time was 30 min. Values are means ± SD of three replicates.

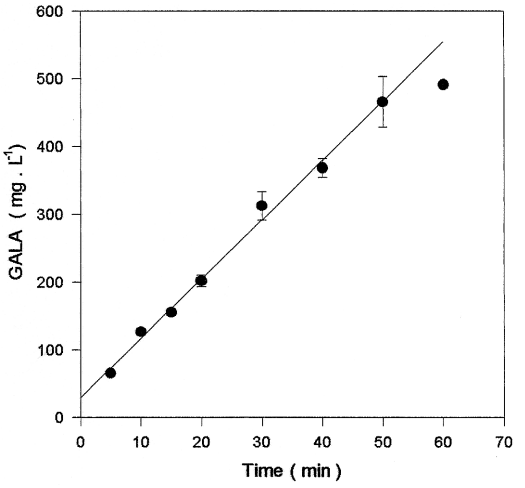

Figure 2 Time course of the GALA released from 20 mg protopectin (mesh 60) by PPase-SE (50 μL enzyme stock solution). Values are means ± SD of three replicates.

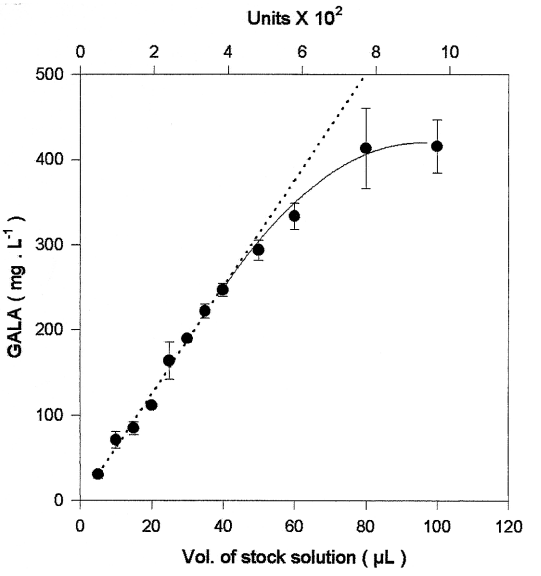

Figure 3 Relationship between enzyme activity (as GALA released from mesh 60 protopectin) and amount of PPase-SE (as the volume of stock enzyme solution and as enzyme units in the reaction mixture). Reaction time was 30 min. Values are means ± SD of three replicates.

The mesh 60 fraction protopectin was selected as substrate for further experiments as, among those fractions belonging to the second region of the curve, it was the most abundant obtained in the preparation of the substrate.

Effect of reaction time

Enzyme reaction was carried out at different incubation times (Fig. 2). Reaction rate remained constant up to 50 min of incubation and then decreased. Therefore, an incubation of 30 min was chosen for practical reasons to be used in further experiments.

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 method (Fig. 3). GALA released remained proportional to the quantity of enzyme used up to 50 μL of enzyme stock solution in the reaction test tube.

Proposed method for PPase activity determination

According to the above results, we propose to use 20 mg lemon protopectin (mesh 60) as substrate and a reaction time of 30 min for PPase activity evaluation. Under these reaction conditions, one unit of PPase activity is defined as the activity that releases soluble pectic substances corresponding to the reducing power of 1 μmol of GALA per minute. Therefore, the maximal amount of enzyme activity added to the test tube should not exceed 0.045 PPase units (Fig. 3).

Quantification of PPase activity in culture samples

Different dilutions (1/1, 1/1.5, 1/2 and 1/3) of a culture supernatant of *Geotrichum klebahnii* ATCC 42397 were used in order to test if this technique was appropriate for practical purposes. Results are shown in Table 1. As it can be seen, no significant differences were observed among tested dilutions.

Conclusions

According to the above results, particle size has a marked influence on enzyme activity and should be carefully considered for the quantification method of this type of enzymes. Standardisation of the assay method is very difficult due to the variability of the substrate

Table 1 PPase activity (units.mL^{-1}) determined by using the final method proposed, in different dilutions of a culture supernatant of Geotrichum klebahnii ATCC 42397. See text for details

Supernatant dilution	Measured activity ($\times 10^2$)	Recovery (%)
1/1	3.66	100 (1.2)
1/1.5	2.26	92.6 (4.5)
1/2	1.65	89.9 (9.6)
1/3	1.17	96.2 (8.9)

Values are means of three replicates. The standard deviation (in parentheses) is expressed as a percentage of the average.

(e.g. type of starting plant tissue, degree of ripeness, procedure for protopectin preparation, etc.). Nevertheless, it is highly advisable to follow the present assay methodology in those laboratories dealing with this type of enzymes. On the other hand, our results suggest that further research is needed in order to evaluate the influence of the actual surface area of the substrate available to the enzyme.

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