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Polygalacturonase Activity in Starfruit

H. M. Ghazali & C. K. Leong

Department of Food Science, Universiti Pertanian Malaysia,
43400 UPM, Serdang, Selangor, Malaysia

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ABSTRACT

*Polygalacturonase (PGase) was extracted and partially purified from ripe starfruit (*Averrhoa carambola, *L.). 2·3- and 7·8-fold purifications were obtained following purification by Amicon ultrafiltration and Sephadex G-100 filtration, respectively. The apparent pH optimum of PGase in the Amicon concentrate and gel filtrate (combined fractions 6–8) was 5·2. Viscometric and reductometric analyses, on PGase in the gel filtrate and paper chromatographic analysis on the enzyme reaction mixture, were carried out to ascertain the mode of action. The results obtained indicated that exoPGase was the only PGase enzyme present in ripe starfruit.*

INTRODUCTION

Polygalacturonase (PGase) has been implicated in the softening of many fruits such as tomatoes (Hobson, 1964; Grierson *et al.*, 1981), peaches (Pressey & Avants, 1971), pears (Pressey & Avants, 1976), avocado (Reymond & Phaff, 1965; Zauberman & Schiffmann-Nadel, 1972) and mangoes (Roe & Bruemmer, 1981). The enzyme is essentially a hydrolase and the modes of action are attributed to two enzymes, exoPGase (EC 3.2.1.15) and endoPGase (EC 3.2.1.40). ExoPGase acts by removing galacturonic acid units from the non-reducing ends of polygalacturonic acid (PGA) while endoPGase hydrolyses PGA randomly, giving rise to short chain oligogalacturonic acids which can ultimately be hydrolysed to a mixture of trigalacturonic, digalacturonic and monogalacturonic acids, depending on the enzyme considered. Fruits such as peaches (Pressey & Avants, 1973*a*), tomato (Pressey & Avants, 1973*b*; Tucker *et al.*, 1980),

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papaya (Chan & Tam, 1982) and cucumber (Pressey & Avants, 1975*a*; McFeeters *et al.*, 1980) were found to have both enzymes. Avocado (Reymond & Phaff, 1965) and carrot (Pressey & Avants, 1975*b*), on the other hand, have only one PGase enzyme each, endoPGase and exoPGase, respectively. There has been no report on the PGase of starfruit (*Averrhoa carambola*, L.), a fruit that is gaining popularity in this region. In this study, we report the extraction, purification and mode of action of PGase of ripe starfruit.

MATERIALS AND METHODS

Extraction and Purification of Starfruit PGase

Ripe starfruits with orange–yellow surface colouration were bought from an orchard in Serdang on the eve of extraction and kept in the cold room (4°C) before they were used. Extraction and purification of PGase were carried out at 2–4°C.

500 g of cleaned and sliced starfruit were homogenised in a blender with 500 ml of cold 50 mM potassium phosphate buffer, pH 7·5, containing 6% (w/v) $(NH_4)_2SO_4$, 0·5% (v/v) Triton X-100 and 1·5% (w/v) poly-(vinylpyrrolidone) (PVP). The composition of the extraction medium was based on that of Riov (1975) and Galeazzi *et al.* (1981). The homogenate was stirred for 30 min and then centrifuged for 30 min at 11 000 rpm using a refrigerated centrifuge (Sorvall RC-5B, Dupont Instrument). The pellet was discarded and PGase was precipitated from the supernatant with $(NH_4)_2SO_4$ to 80% saturation. The addition of the salt resulted in the formation of clumps of gummy material that floated in a clear yellowish solution, instead of a precipitate. The mixture was stirred for 1 h and then filtered through cotton wool. The filtrate was discarded and the residue dissolved in 150 ml of 1% NaCl solution. A yellowish orange slurry was formed since not all of the residue was soluble. The slurry was filtered through a Whatman No. 1 filter paper that was pre-layered with PVP and the filtrate was then dialysed overnight against 1% NaCl solution. The dialysate was clarified by centrifugation at 15 000 rpm for 20 min before concentration to 1/3 the volume by Amicon ultrafiltration using a YM-10 membrane. Compressed air was used to give a working pressure of 50 psi.

Further purification of PGase was achieved by gel filtration on Sephadex G-100. A 10-ml aliquot of the Amicon concentrate was applied to a column (50 × 2 cm inside diameter) containing gel that has been equilibrated with 1% NaCl solution. Elution was carried out also using 1% NaCl.

Each 10-ml fraction collected was further concentrated by Amicon ultrafiltration using a YM-10 membrane to one-third its volume before assaying for PGase activity. Absorbance of non-concentrated fractions was measured at 280 nm.

Assay for PGase activity

PGase activity was determined reductometrically according to Pressey & Avants (1976). The reaction mixture consisted of 0·5 ml of enzyme fraction, 0·5 ml of 50 mM Tris-acetate buffer (pH 5·2) and 1·0 ml of 1% sodium polypectate (Sigma Chemical Co.) solution. The pH of the substrate solution was also 5·2. A reaction blank which contained 0·5 ml of enzyme fraction that had been heated in a boiling water bath for 3 min, 0·5 ml of the buffer and 1·0 ml of substrate, was run with each experiment. The reaction mixtures and blanks were incubated at 37°C for 24 h, after which the reaction was terminated by heating in a boiling water bath for 3 min. The increase in reducing groups was measured by the arsenomolybdate method (Nelson, 1944; Southgate, 1976) using galacturonic acid as standard. One unit of PGase was defined as the amount of enzyme which produces 1 μmol reducing groups (RG) per millilitre of enzyme per 24 h. Specific activity was expressed as units of enzyme per milligram of protein in the enzyme solution.

Protein determination

The protein content of the enzyme fractions were determined by the modified Lowry method using crystalline bovine serum albumin as the standard protein (Lowry *et al.*, 1951; Eggstein & Kreutz, 1967).

pH profile

The pH profiles of PGase were obtained by assaying the activity in the pH range of 2·8 to 7·6 for the Amicon concentrate and combined fractions 6, 7 and 8 for the gel filtrate. 50 mM Tris-acetate buffer was used to obtain the pH range.

Mode of PGase action

Viscometric–reductometric analyses

For this study, viscometric and reductometric assays were carried out simultaneously on the combined gel filtration fractions 6, 7 and 8. A total of 50 ml of the same reaction mixture (pH 5·2) as described under the section above headed 'Assay for PGase activity' was used. Viscometric assay was carried out using an Ostwald viscometer where flow times were measured at

37°C. PGase activity by the viscometric procedure was expressed as per cent viscosity loss and fluidity (Chan & Tam, 1982; Tam, 1983). The Comparison Ratio (CR) value, used for determining the mode of PGase action, was obtained by dividing the rate of increase in fluidity by the rate of increase in reducing groups (Tam, 1983).

Paper chromatography

Descending paper chromatography was used to identify the end products of polypectate degradation by PGase. 20 ml of the standard reaction mixture was incubated at 37°C and 2 ml were withdrawn at regular intervals, heated in a boiling water bath for 3 min and 10–20 μl were chromatographed on Whatman No. 3 MM paper. Galacturonic acid and sodium polypectate served as standards. The running solvent was either ethyl acetate–acetic acid–H_2O (10:5:6 v/v) or butanol–acetic acid–H_2O (4:2:3 v/v) and the reaction products were detected using aniline hydrogen phthalate reagent.

RESULTS AND DISCUSSION

Extraction and purification of PGase

Preliminary studies showed that no PGase activity was detected in the crude extract and after $(NH_4)_2SO_4$ precipitation when the extractant solution used did not contain PVP. When PVP was added to the extractant medium, detectable PGase activity was found in the $(NH_4)_2SO_4$ extract and more purified extracts but not in the crude extract. The absence of a detectable level of PGase in extractant medium without PVP can possibly be attributed to the presence of polyphenolic compounds and other interfering substances in the fruit which caused inactivation of the enzyme. PVP has been often used to improve the enzyme preparations obtained from fruit materials (Jones *et al.*, 1965; Loomis & Battaile, 1966).

Table 1 shows the results of the purification of PGase from ripe starfruit. Since PGase activity was not detected in the crude enzyme fraction, PGase activity of the $(NH_4)SO_4$ dialysate was taken as a reference in calculating the purification fold. For the Amicon concentrate, a 2·3-fold purification was obtained while a value of 7·8 was achieved following gel filtration.

pH profile

The pH profile of PGase using the Amicon concentrate is shown in Fig. 1. A similar profile and apparent pH optimum was also obtained when a

TABLE 1
Purification of Starfruit Polygalacturonase

Fraction	*Volume (ml)*	*Protein conc. (mg/ml)*	*Activity (units)*	*Specific activity (units/mg protein)*	*Purification fold*
Crude extract	1150	3·20	ND	—	—
$(NH_4)_2SO_4$ precipitate dialysate	132	0·97	1·61	1·66	1·0
Amicon concentrate	40	1·23	4·53	3·68	2·2
Gel filtrate, fraction 7	10	0·36	4·62	12·80	7·7

ND, not detectable.

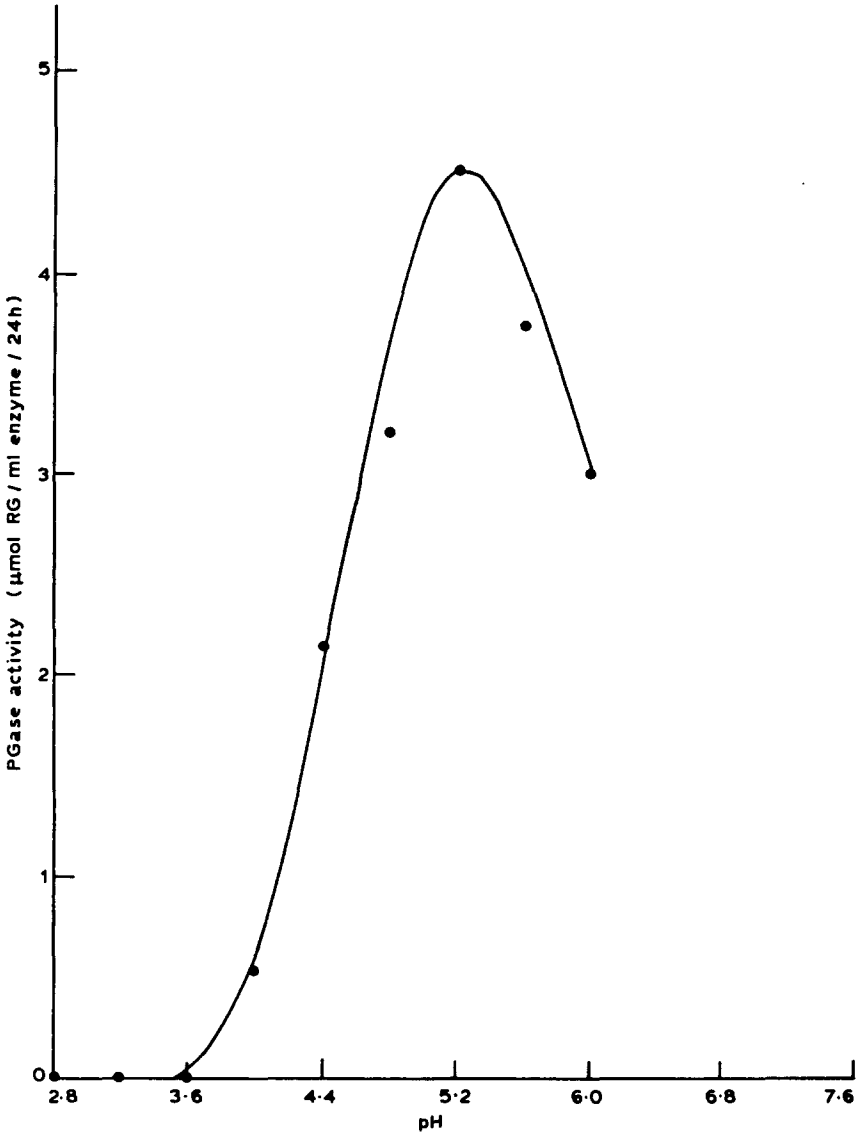

Fig. 1. pH profile of PGase in the Amicon concentrate when assayed using 50 mM Tris-acetate buffer.

combination of gel filtrate fractions 6–8 was used. It was observed that when the enzyme assay was carried out at pH > 6 for the Amicon concentrate and pH > 6·8 for the gel filtrate, the reductometric absorbance for the unheated reaction mixtures was lower than the absorbance for the heated reaction mixtures and was the same as the diluent blanks that contained buffer, diluent (1% NaCl solution) and substrate only. There was no difference in the absorbance of heated mixtures at all pH values. The

absorbance of the diluent blank was due to the presence of substrate while that of the heated reaction blank was due to the substrate and substances such as protein present in the enzyme extract. Both the substrate and protein are capable of complexing with Cu^{2+}. The trend was the same even when the buffer was changed from Tris-acetate to Tris-maleate. It was also observed that, at these pHs, the unheated reaction mixtures contained insoluble particles that remained in suspension at the end of the incubation period.

There may be several factors that can give rise to the observations described above. Co-polymerisation between proteins and substances present in the enzyme extract, such as the polyphenolic compounds, could have given rise to the insoluble particles which were in a form that was inaccessible to complexation with Cu^{2+}. Uronic acid oxidase (UAO), the causal agent in a similar observation by Riov (1974) who studied PGase in citrus leaf explants, may also be present in the starfruit enzyme extract. UAO oxidises galacturonic acid into galactaric acid, a non-reducing sugar acid, in the presence of water and molecular oxygen. Other unknown factors may be operating also. As such, the results obtained could possibly be due to an interplay between these factors but the relative importance of each of these factors in contributing to the above results has yet to be ascertained.

Although the optimum pH was obtained at pH 5·2, this pH is only an apparent pH optimum and may not reflect the actual starfruit PGase pH optimum because the factors that affected the absorbance of unheated reaction mixtures at pH > 6 may also be at work at lower pHs.

Mode of PGase action

When PGase activity was measured on Sephadex G-100 filtrates at pH 5·2, only one peak was obtained with the maximum activity in the fraction 7 (Fig. 2). Further studies were conducted on the combined gel fractions (6–8) in order to determine the mode of action of starfruit PGase and the results obtained are shown in Fig. 3. The two modes of PGase attack on PGA are terminal cleaving and random splitting and these can be differentiated by comparing the rate of substrate size reduction (increase in fluidity through random cleavage of substrate) with the rate of hydrolysis (increase in reducing groups through chain cleavage). The Comparison Ratio (CR) method (Tam, 1983) for computing the results obtained afforded a means of differentiating the two PGase enzymes. Following this method, the CR for starfruit PGase in the gel filtrate was calculated to be 0·15, a value that is indicative of a predominant presence of exoPGase (Chan & Tam, 1982; Tam, 1983).

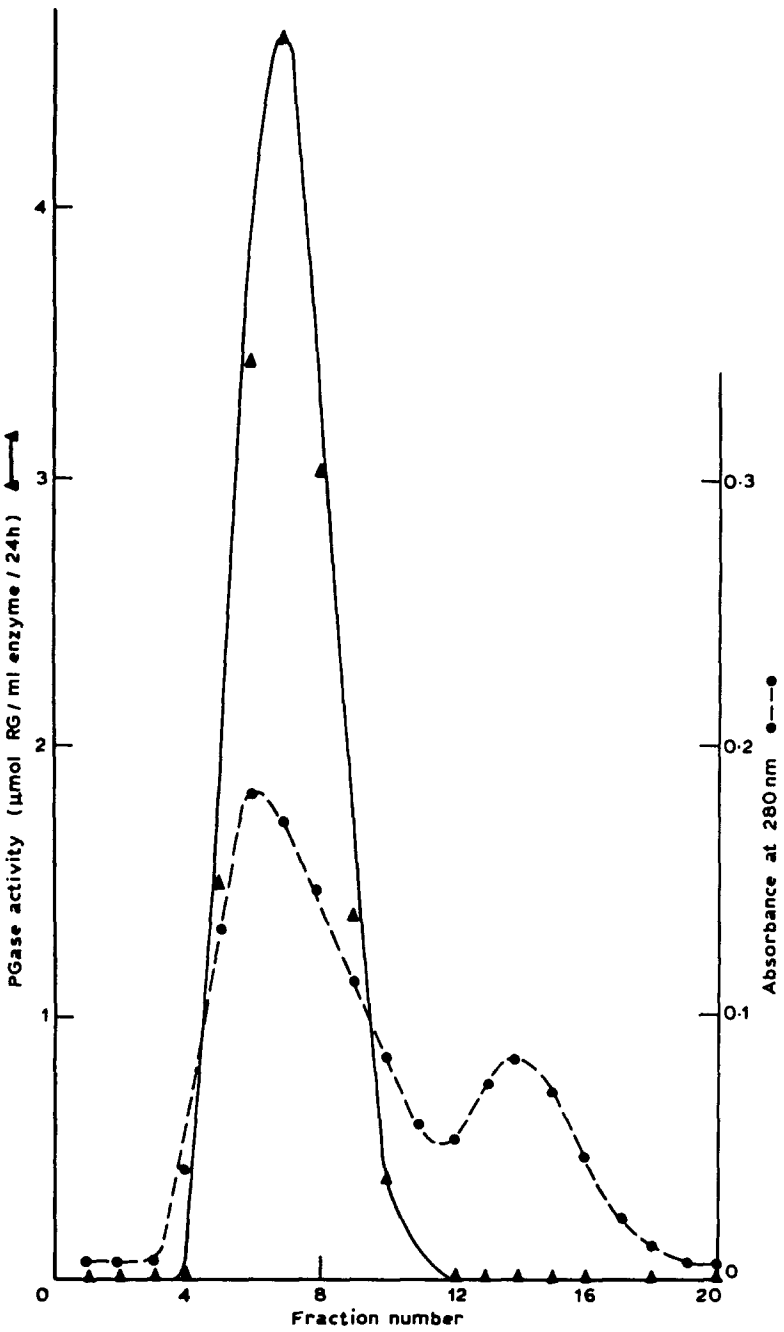

Fig. 2. Sephadex G-100 elution profile of PGase.

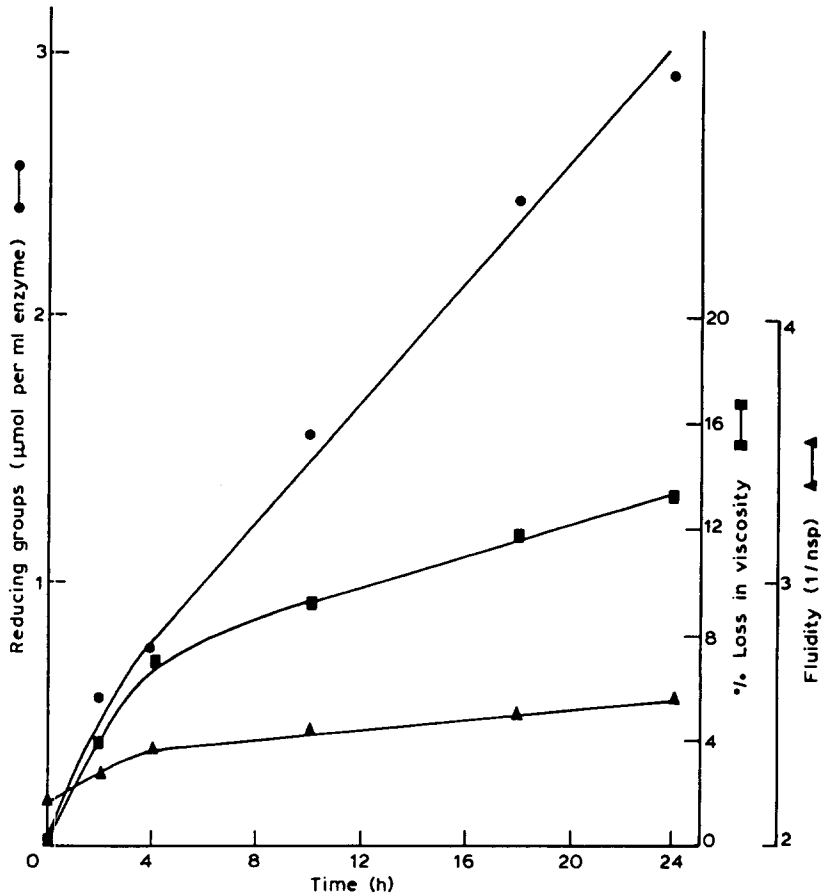

Fig. 3. PGase activity in terms of reducing groups formed, fluidity and per cent loss in viscosity with respect to incubation time.

Paper chromatographic analysis of the enzyme reaction mixture lent further support for the presence of exoPGase, since galacturonic acid was identified as the sole product of polypectate breakdown. The analysis gave rise to a single mobile spot that was visible from the fourth hour onwards. The R_f value of this spot corresponded to that of the galacturonic acid standard and the intensity of the spot increased with increasing reaction time.

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