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Purification and characterization of polygalacturonase from banana fruit

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Abstract

Polygalacturonase isoenzyme 3 (PG-3) was purified to homogeneity with a specific activity of 0.7 μ katal mg^{-1} protein from banana fruit pulp. The purified enzyme was a glycoprotein with ca. 8% carbohydrate. The molecular weight of the native enzyme was found to be 90 ± 10 kDa with a subunit molecular weight of 29 ± 2 kDa. The enzyme exhibited optimum activity at pH 4.3 and temperature 40°C with activation energy 35.4 kJ mol^{-1}. A unique property of the enzyme was the requirement of –SH groups for the enzyme activity. The enzyme was inhibited by p-CMB and activated by 2-ME and DTT. The inhibition of p-CMB could be reversed by DTT. The enzyme contained eight free –SH groups. The K_m of the enzyme was 0.15% for polygalacturonic acid.

Keywords: *Musa acuminata*; Musaceae; Banana; Enzyme purification; Isoenzyme; Polygalacturonase

1. Introduction

Polygalacturonase (PG) catalyzes the hydrolytic cleavage of α(1→4) galacturonan linkages of pectin. PG has been isolated from tomato (Hobson, 1965; Tucker et al., 1980; Brady et al., 1983), avacado (Awad and Young, 1979), apple (Bartley, 1978), pear (Ahmed and Labavitch, 1980), strawberry (Nogata et al., 1993) and other fruits. Dominguez-Pulgjaner et al. (1992) observed a dramatic increase in two polypeptides of 28 and 42 kDa during ripening of banana fruits and suggested that the 42 kDa polypeptide could be PG related proteins as they cross-reacted with tomato PG antibodies. The high phenolics and starch content of banana fruit has made protein extraction and quantitative measurement of enzyme activities difficult (Dominguez-Pulgjaner et al., 1992; Medina-Suárez et al., 1997). We were successful in demonstrating and isolating multiple forms of PG from banana fruits (Pathak and Sanwal, 1998), using a Triton X-100 supplemented homogenizing medium. The endo enzyme PG-1 was found to decrease during the ripening of banana but endo enzyme PG-3 increased progressively during ripening of banana fruits. We now report the purification of PG-3 from ripe banana fruits, its properties and physico-chemical characterization.

2. Results and discussion

2.1. Purification of polygalacturonase-3

The summary of the enzyme purification from ripe banana fruit pulp is given in Table 1. The DEAE cellulose chromatography followed by Sephadex G-150 chromatography (Fig. 1a) led to a 41-fold purification of the enzyme from the initial extract. A single peak of enzyme activity was observed on Sephacryl S-200 chromatography (Fig. 1b). The active pooled fraction was enriched 78-fold from the initial extract. The enzyme

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Table 1
Purification of polygalacturonase-3 from ripe banana fruits

Fraction	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Fold enrichment
Initial extract	750	862	1230	0.71	100	1
Ammonium sulfate (0–90%)	100	754	1140	0.66	87	0.9
DEAE-cellulose	60	196	25	7.84	23	11
Sephadex G-150	15	72	2.5	28.8	8	41
Sephacryl S-200	12	67	1.2	55.8	7.7	78
Con-A Sepharose	8	59	0.3	196.7	6.8	277
Q-Sepharose	2	49	0.2	245.0	5.7	345

was further purified by affinity chromatography on Con-A Sepharose column. The enzyme could be eluted by 0.1 M methyl mannoside (Fig. 1c) resulting in 277-fold purification. The enzyme, subjected to Q-Sepharose chromatography, could be eluted in 0.75 M NaCl (Fig. 1d), resulting in overall purification of the enzyme by 345-fold with 5.7% recovery from the initial extract. Tucker et al. (1980) purified the low molecular weight PG-2 isoenzyme from tomato fruits to homogeneity and reported a specific activity of about 0.1 μ katal mg^{-1} protein. Ali and Brady (1982) purified PG of tomato fruits to homogeneity and recorded specific activities in the range 0.1-1.7 μ katal mg^{-1} protein. In the present investigation PG-3 isoenzyme from banana fruits has been purified with a specific activity of 0.25 μ katal mg^{-1} protein. In the presence of 5 mM DTT, the specific activity of PG-3 was found to be ca. 0.7 μ katal mg^{-1} protein which compares with the value reported by Ali and Brady (1982) and is better than reported by others. The purified enzyme gave one protein band on native PAGE at pH 8.3 on silver staining and exhibited no activity for endopectate lyase and pectin methyl esterase (PME) (data not shown). Complete loss of PG activity occurred in 3 weeks when the purified enzyme was stored frozen.

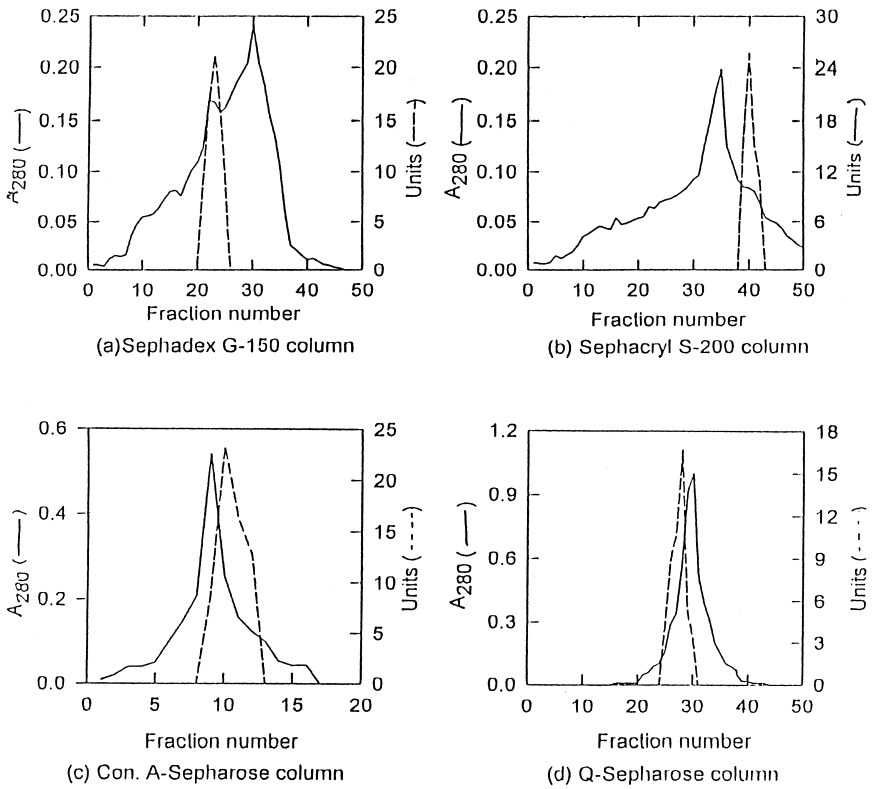

Fig. 1. Chromatographic profiles of resolved proteins of DEAE-cellulose fraction (0.70–0.85 M NaCl) obtained from banana fruit pulp. Details are given in Section 3.

2.2. Carbohydrate content

PG-3 is a glycoprotein as is evident from the carbohydrate content of the purified enzyme and it being stained by Schiffs base reagent. The carbohydrate content (glucose equivalent) was 8% of the enzyme. The enzyme was found to get bound to lectin containing Sepharose. The enzyme preparation bound to Con-A Sepharose could be eluted with 100 mM methyl mannoside. Ali and Brady (1982) and Moshrefi and Luh (1983) reported glycoprotein nature of PG isolated from tomato fruit.

2.3. Properties of PG-3 enzyme

The enzyme activity showed linearity with time upto 60 min and linearity with enzyme concentration upto 15 μg protein. PG-3 exhibited optimum activity at pH 4.3.

2.4. Effect of temperature

The effect of temperature was studied in the range of 20–45°C , keeping the reaction time to 30 min. The enzyme activity increased with the rise in temp. and exhibited optimum activity at 40°C. The Arrehenius plot of data shows energy of activation of 35.4 kJ mol^{-1} for PG-3, which was almost half of that reported for tomato PG-I and PG-II (Moshrefi and Luh, 1983).

Table 2
Effect of sulfhydryl binding reagents and sulfhydryl agents in PG-3 activity

Treatment	Concentration (mM)	Activity[a] (U/ml)	Activation (+), Inhibition (−) (%)
Nil		21.3 ± 1.95	—
Iodoacetamide	1	17.1 ± 1.45	(−) 20
p-CMB	0.1	11.8 ± 0.95	(−) 45
2-ME	1	30.8 ± 2.50	(+) 45
	5	36.3 ± 2.85	(+) 70
	10	31.0 ± 2.65	(+) 46
DTT	1	29.8 ± 2.60	(+) 40
	3	44.7 ± 3.50	(+) 110
	5	59.6 ± 4.05	(+) 180
	7.5	26.2 ± 1.95	(+) 23
	10	21.2 ± 1.85	(−) 1
p-CMB	0.5	7.4 ± 0.55	(−) 65
p-CMB + DTT	0.5 + 1	10.5 ± 0.80	(−) 51
	0.5 + 5	14.2 ± 1.15	(−) 33
	0.5 + 7.5	16.5 ± 1.50	(−) 23
	0.5 + 10	20.0 ± 1.95	(−) 06

[a] Value is mean ± standard error of three experiments.

2.5. Effect of sulfhydryl binding reagents and sulfhydryl agents

The sulfhydryl binding reagents, iodoacetamide and p-CMB inhibited the enzyme activity, while sulfhydryl agents, DTT and 2-ME stimulated the PG-3 enzyme activity (Table 2). The PG-3 activity increased progressively with the increase in the concentration of DTT and maximum activation of 180% was observed in 5 mM concentration. Further increase in DTT concentration resulted in decreased activation. Another sulfhydryl agent 2-ME also activated PG-3 activity with 70% activation in 5 mM concentration. Further increase in concentration to 10 mM produced less activation.

To study the reversal of inhibition of the enzyme activity by p-CMB, different concentrations of DTT were added to an aliquot of the enzyme preparation which had been inhibited 65% by 5 min contact with 0.5 mM p-CMB. The mixture was preincubated for 5 min at 37°C and then the enzyme activity determined. The reversal of p-CMB inhibition by increasing concentrations of DTT is evident from Table 2. The increased PG activity on incorporation of sulfhydryl agents (DTT, 2-ME) lends support to the need for sulfhydryl groups for the functioning of the enzyme. The inhibition of PG by p-CMB and iodoacetamide suggests the likely presence of SH-group in the active center of the enzyme. The involvement of SH groups in the enzyme activity is further substantiated by reversal of p-CMB inhibition by DTT.

The number of sulfhydryl groups in the enzyme molecule was determined by titration against DTNB. Calculation based on increased A_{412} shows eight free –SH groups per mol of the enzyme. No increase in A_{412} was observed on addition of 1% SDS. It appears that there are no buried sulfhydryl groups. To the best of our knowledge, it is the first example of a sulfhydryl

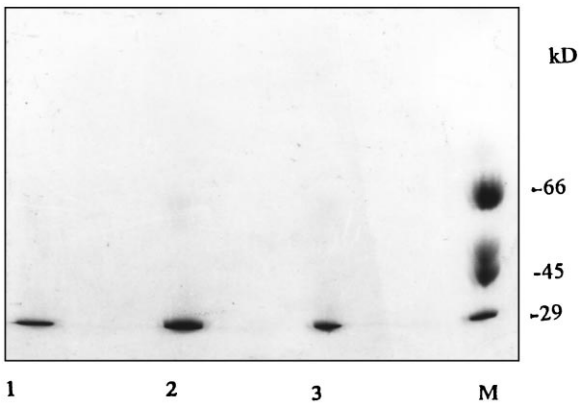

Fig. 2. SDS-PAGE of PG from banana fruit pulp, using 10% gel at pH 8.5. 1: PG (5 μg); 2: PG (10 μg); 3: Carbonic anhydrase; M: Marker proteins.

containing PG in higher plants. Ali and Brady (1982) found that PG enzymes in tomato fruits were unaffected by thiol reagents.

2.6. Molecular weight

The gel filtration chromatography of the purified enzyme on Sephadex G-150 indicated M_r of 90 ± 10 kDa. SDS-PAGE of the purified enzyme shows homogeneity of the enzyme preparation with migration equal to carbonic anhydrase (Fig. 2). The subunit M_r of PG was found 29 ± 2 kDa from the plot of mobilities of marker proteins. Tucker et al. (1980) reported M_r of 100 and 42 kDa for PG-1 and PG-2 from tomato fruit, both giving rise to subunit M_r of 46 kDa.

2.7. Saturation curve with purified polygalactouronic acid

The substrate saturation curve for PG-3 was hyperbolic when purified polygalactouronic acid (PGA), consisting of higher chain polymers was employed. The substrate saturation curve was found biphasic with unfractionated PGA. It may be possible that lower chain polymers of PGA acted as inhibitors of PG. The Lineweaver–Burk's data indicate K_m value of 0.15% for PGA.

It has been suggested that PG is primarily responsible for pectin degradation and fruit softening (Hobson, 1965; Brady et al., 1983). PME is reported to increase in banana as ripening proceeds (de Swardt and Maxie, 1967). This is likely to increase polygalacturonides, the de-esterified pectin molecule, which is the substrate for PG. The isoenzyme PG-3 increases in banana during ripening (Pathak and Sanwal, 1998). Dominguez-Pulgjaner et al. (1992) also observed an increase in a polypeptide, 42 kDa, during ripening in banana, which cross-reacted with tomato PG antibodies, along with a polypeptide of 28 kDa. In the present investigation we find the subunit molecular weight of PG-3 closer to 28 kDa polypeptide. Thus, there is ample evidence that PG increases during ripening of banana fruit. The experiments on transgenic tomato indicate that fruit softening is not affected significantly in tomatoes with down-regulated PG activity (Smith et al., 1990). Expression of a PG transgene in tomato ripening mutant rin resulted in pectin solubilization and depolymerization at near wild-type levels, but fruit softening did not take place (Giovannoni et al., 1989; DellaPenna et al., 1990). Recent molecular evidence suggests that pectate lyase is abundant in banana fruits. Dominguez-Pulgjaner et al. (1997) have isolated a cDNA clone which is specifically expressed in ripe banana fruit and has homology to pectate lyase. Medina-Suárez et al. (1997) also observed clones with homology to pectate lyases abundant in banana pulp. It appears that in addition to PG and PME, pectate lyases are likely to be involved in softening associated with ripening in banana fruit.

3. Experimental

3.1. Fruit tissue

Ripe banana fruits (*Musa acuminata* cv Harichhal) Genome AAA were collected from local market.

3.2. Purification of polygalacturonase

All steps were carried out in cold (4–6°C). Initial steps of purification of the enzyme were same as described earlier (Pathak and Sanwal, 1998). Banana fingers were freed from peel, sliced longitudinally and the central core carrying seeds cut out and discarded. The pulp was cut into small pieces and homgenized in a Waring blender with cold homogenizing medium consisting of 0.02 M Na–Pi buffer, pH 7.0, 0.02 M neutralized EDTA, 1% Triton X-100, 0.02 M cysteine–HCl and 1 mM PMSF for 2 min to give 30% homogenate. The homogenate was centrifuged at $15{,}000 \times g$ for 15 min to yield the initial extract. $(NH_4)_2SO_4$ was added to 0.9 sat, the ppt. suspended in 0.02 M Na–Pi buffer of pH 7 containing 1 mM PMSF and dialysed against the same buffer overnight. The dialysed suspension was centrifuged at $15{,}000 \times g$ for 15 min and the clear supernatant loaded onto a DEAE-cellulose column (3.2×24 cm), pre-equilibrated with 0.02 M Na–Pi buffer of pH 7. The column was washed with 3 bed vol of the above buffer and 8 ml fractions collected. The adsorbed proteins were collected using a linear NaCl gradient (0–1 M). The active frs eluted between 0.70 and 0.85 M NaCl were pooled and concentrated with sucrose. The enzyme was then applied to a Sephadex G-150 column (1.6×80 cm), pre-equilibrated with 0.02 M Na–Pi buffer, pH 7, and proteins eluted with the same buffer. The enzyme was further purified by gel filtration over Sephacryl S-200 column (1.6×95 cm), pre-equilibrated with 50 mM Tris–HCl buffer, pH 7.5 containing 0.25 M NaCl, frs of 3 ml were collected. The active frs were pooled, concentrated by dialysis against sucrose and purified by affinity chromatography through Con A-Sepharose column (1×6 cm), which had been pre-equilibrated with the binding buffer (20 mM Tris–HCl, pH 7.4, containing 0.5 M NaCl). The enzyme was eluted with two bed vol of the binding buffer, one bed vol of 0.1 M α-D-methyl mannoside and one bed vol of 0.2 M α-D-methyl mannoside. Frs of 2 ml were collected. Further purification of the enzyme was achieved by anion exchange chromatography on Q-Sepharose.

The enzyme was passed through Q-Sepharose column (1 × 6.5 cm) which had been pre-equilibrated with 50 mM Na–Pi buffer of pH 7.2 and eluted with 2 bed vol of the above buffer containing 0.5, 0.75 and 1 M NaCl, respectively. The enzyme was eluted in 0.75 M NaCl.

3.3. Purification of polygalacuronic acid

PGA (Sigma) was fractionated by gel filtration on a Sepharose CL 4B column (1.5 × 80 cm) using 100 mM acetate buffer (pH 5.5). Dextrans 10 and 70 kDa were used as markers. PGA (8 mg) was dissolved in 2 ml buffer and eluted with the same buffer. Frs of 2 ml were collected and analysed for uronic acid content. Peak frs collected between 70 kDa and void volume were pooled and lyophilized.

3.4. Enzyme assay

PG activity was assayed as described earlier (Pathak and Sanwal, 1998). The formation of reducing groups was measured by DNS method (Miller, 1959). One unit (U) of the enzyme catalyzed the liberation of one nmole of galacturonic acid in 1 s under the conditions of the enzyme assay. Specific activity was expressed as U per mg protein.

3.5. Protein determination

Protein determination was carried out according to the method of Lowry et al. (1951), using BSA as a standard.

3.6. Carbohydrate content

The phenol sulfuric method (Dubois et al., 1956) with glucose as a standard was used to determine carbohydrate in the purified enzyme preparation.

3.7. Molecular weight determination

The molecular weight of the purified enzyme was determined by gel filtration chromatography on Sephadex G-150 column according to the method of Andrews (1964), using β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 29,000) and cytochrome c (M_r 12,400) as standard proteins.

3.8. Polyacrylamide gel electrophoresis

Electrophoresis was carried out according to the method of Laemmli (1970), but without SDS, using 8% gel. Protein bands were visualized using silver staining (Blum et al., 1987). SDS-PAGE was carried out according to the procedure of King and Laemmli (1971) employing 10% polyacrylamide in 0.2 M Tris–HCl, pH 8.5, with 1 mM EDTA and 0.1% SDS. The electrophoresis was carried out in Tris–Glycine buffer (pH 8.5) containing 0.1% SDS by passing a current of 30 mA until the sample ran into the stacking gel and then 60 mA until the dye front migrated to within 2 cm of the bottom of the gel. Carbonic anhydrase (SU M_r 29,000), ovalbumin (SU M_r 45,000) and bovine serum albumin (SU M_r 66,000) were used as marker proteins.

3.9. Determination of sulfhydryl groups

The sulfhydryl groups were determined according to method of Ellman (1959) using DTNB. The number of sulfhydryl groups reacted was calculated using molar extinction coefficient of DTNB as 13,600 M^{-1} cm^{-1}. To determine the total number of sulfhydryl groups present in the protein, the reaction with DTNB was further continued after addition of SDS to a final concentration of 1% until no more change in absorbance was observed.

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