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MULTIPLE FORMS OF POLYGALACTURONASE FROM BANANA FRUITS

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Key Word Index—*Musa acuminata*; Musaceae; banana; pectin degradation; polygalacturonase; multiple forms; fruit ripening.

Abstract—Three multiple forms of polygalacturonase (PG) in ripe and two in unripe banana (*Musa acuminata*) fruits were separated by DEAE-cellulose and further purified using Sephadex G-150 chromatography. The multiple forms can be differentiated from each other on the basis of their properties. PG1 and PG3 were identified as endo-PG and PG2 as exo-PG on the basis of decrease in viscosity, increase in reducing sugar and the reaction product. PG2 and PG3 increased with the ripening of fruits. PG1, PG2 and PG3 exhibited optimum activity at pH 3.3, 3.7 and 4.3, respectively. Complete loss of PG2 and PG1 activities occurred at 60 and 70°, but PG3 retained 60 and 50% activity respectively. The three forms showed a different response towards divalent metal ions. Ca^{2+} activated PG1 activity only. Teepol 0.1%, inhibited PG1 activity by 25%, but PG2 and PG3 activities were completely inhibited. CTAB, 0.1%, had no effect on PG1 and PG2 activities, but inhibited PG3 activity by 40%. 2-ME stimulated PG2 and PG3 activities but had no effect on PG1 activity. Gel filtration through Sephacryl indicated M_r of 23,200, 58,000 and 130,000, respectively, for PG1, PG2 and PG3. The substrate saturation curve for PG1 and PG2 were Michaelian, while PG3 showed biphasic curve. The *Km* values of PG1 and PG2 were 0.22% and 0.14%, respectively.

INTRODUCTION

Ripening of fruit is associated with textural changes and extensive softening of the tissues. Microscopic examination of the cells from banana show cell wall modifications in cells of the pulp during ripening of banana [1]. In many fruits, the most apparent changes occur in wall pectic polysaccharides, which may become more soluble and show a reduction in M_r [2, 3]. Comparatively little information is available from banana. Soluble pectins are depolymerized to some extent during ripening of banana [4]. Changes in pectin structure indicate that pectin degrading enzymes may be active in these cell walls. Pectin methyl esterase (EC 3.1.1.11) activity has been identified in banana tissue [5–7] and reported to exist in two isoforms [7]. Polygalacturonase (PG) as either the endo-acting (EC 3.2.1.15) or as exo-acting (EC 3.2.1.67) enzyme has been reported in ripening bananas [8] and other fruits [9–14] and in many cases can be correlated with the softening of fruits. Multiple forms of PG have been demonstrated in strawberry [14], tomato [15–17], peach [18] and pears [19]. No report exists on the role of PG in softening of banana fruits and the presence of multiple forms of the enzyme in banana. The present investigation describes the method for separating the multiple forms of the enzyme, the activities of the multiple forms of PG at different stages of fruit ripening and some of their properties in banana (*Musa acuminata*) fruit pulp.

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RESULTS AND DISCUSSION

Extraction of polygalacturonase enzyme using different media for homogenization

The incorporation of 1% Triton X-100 in the homogenizing medium led to the extraction of 85% of polygalacturonase activity in 15,000 *g*, 30 min supernatant. PVP (1%), on the other hand, led to only 28% extraction of the enzyme activity in 15,000 *g*, 30 min supernatant. Little activity for PG was obtained in absence of Triton X-100 or PVP.

Multiple forms of polygalacturonase at various stages of banana ripening

The total PG activity in the unripe fruit pulp was either absent or so low that it could not be determined.

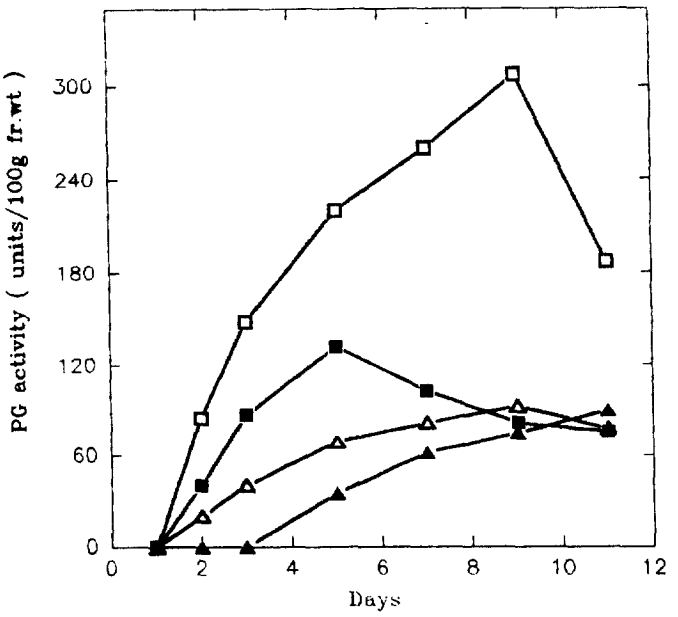

Fig. 1. Activities of polygalacturonase and its multiple forms at various stages of ripening of banana fruits. Total PG, □—□; PG1, ■—■; PG2, △—△; PG3, ▲—▲.

PG activity was first detected on 2nd day and then activity increased progressively with the ripening of the fruit and declined in over-ripened fruit (Fig. 1). Similar observations have been reported in ripening of tomato fruit [9, 12, 20]. The respiratory rate was low in unripe fruit (1 to 3 days). A sharp increase in respiration occurred on the 5th day, indicating a climacteric rise, followed by a decline in respiration.

DEAE-cellulose chromatography of dialysed 0.9 satd ammonium sulfate fraction from unripe (preclimacteric) banana fruit pulp revealed an unadsorbed PG activity, which resulted on washing the column with 3 bed volumes of 0.02 M Na-Pi buffer, pH 7. This activity has been designated here as PG1. The adsorbed protein on elution by linear gradient of 0–1 M NaCl gave one peak of the enzyme activity eluting between 0.10–0.25 M, which has been designated as PG2. DEAE-cellulose chromatography of the ammonium sulfate fraction from ripe (post climacteric) banana pulp again revealed an unadsorbed PG activity corresponding to PG1. The elution of the adsorbed protein by NaCl gradient gave rise to two peaks of the enzyme activity, one eluting between 0.10–0.25 M NaCl, corresponding to PG2, and the other eluting between 0.75–0.95 M NaCl, which has been designated as PG 3 (Fig. 2).

The pattern of multiple forms of PG during different ripening stages is shown in Fig. 1. PG1 activity increased up to the 5th day and then declined. PG 2 activity increased throughout ripening and declined in over-ripened fruit (11th day). PG3 activity was not detected in preclimacteric fruit and appeared only on the climacteric rise on the 5th day. The activity then increased progressively during ripening up to the 11th day. It appears that PG2 and PG3 are associated with the ripening of banana fruits. In ripening tomato fruit PG1 accumulated first, followed by accumulation of PG2A and PG2B [21]. The reason for multiplicity of polygalacturonase in banana fruits is not clear. The possibility of the formation of multiple forms due to proteolysis is less because of the presence of protease inhibitor in the isolation medium.

Partial purification of multiple forms of polygalacturonase from post climacteric banana

The partial purification of three multiple forms of PG from ripe banana fruits is summarized in Table 1. Ammonium sulfate precipitation, DEAE-cellulose chromatography, CM-cellulose chromatography, followed by gel filtration through Sephadex G-150 led to purification of PG1 by 117-fold with 13% recovery from the initial extract. Anion exchange chromatography followed by gel filtration led to purification of PG2 and PG3 by 50-and 34-fold with 4 and 8% recovery respectively, from the initial extract. The partially purified PG1 and PG3 were free from pectin methyl esterase activity, while PG2 had a weak activity for pectin methyl esterase.

Characterization of partially purified multiple forms of polygalacturonase

The multiple forms of PG from banana can be distinguished on the basis of their properties.

pH activity relationship

PG1, PG2 and PG3 exhibited optimum activities at pH 3.3, 3.7 and 4.3, respectively in acetate buffer.

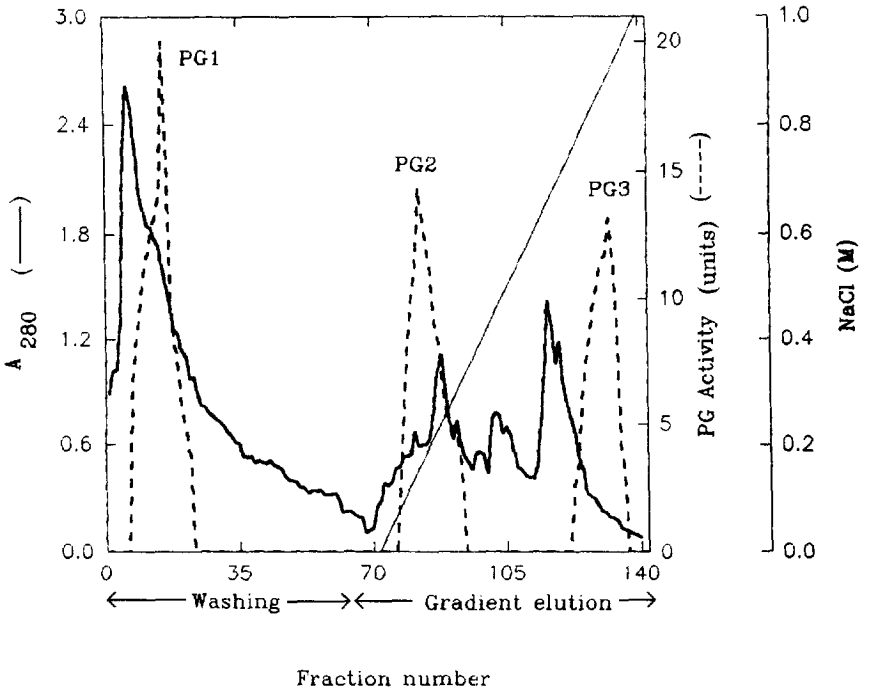

Fig. 2. Separation of multiple forms of polygalacturonase from ripe banana fruits on a DEAE-cellulose column.

Table 1. Partial purification of multiple forms of polygalacturonase from postclimacteric banana fruits

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units mg protein $^{-1}$)	Recovery (%)	Fold enrichment
Initial extract	375	436	615	0.71	100	1.0
Ammonium sulphate (0–90%)	50	392	570	0.69	90	0.9
DEAE-cellulose chromatography						
PG1	104	133	317	0.42	31	0.6
PG2	48	99	18	5.50	23	7.7
PG3	48	88	11	8.00	20	11.2
CM-Cellulose chromatography						
PG1	60	71	17	4.17	16	6.0
Sephadex G-150 chromatography						
PG1	15	58	0.70	82.8	13	117
PG2	9	19	0.54	35.2	4	50
PG3	15	36	1.50	24.0	8	34

Thermal stability

The three multiple forms of the enzyme showed different heat stability when held at various temperature for 5 min. PG3 was heat stable, retaining 60 and 50% activity at 60 and 70°, respectively. PG2 was heat labile with total loss of activity at 60°. PG1 was comparatively less labile, retaining 35% activity at 60°, with total loss of activity at 70°. Differences in heat stability amongst multiple forms of PG have also been reported from tomato [17].

Effect of metal ions

The multiple forms of PG showed different response to divalent metal ions. Mn^{2+} and Mg^{2+} slightly stimulated PG1 activity, had no effect on PG2 activity and slightly inhibited PG3 activity (Table 2). Ba^{2+} did not influence PG1 activity, but slightly inhibited PG2 and PG3 activities. Cd^{2+}, Hg^{2+} and Pb^{2+} inhibited significantly all three forms of the enzyme. Cu^{2+} slightly inhibited PG3, but had no influence on PG1 and PG2 activity. Ca^{2+} significantly activated PG1 activity, but had no effect on PG2 and PG3 activities. EDTA inhibited PG1 activity, but activated PG2 activity. The inhibition of PG1 activity by EDTA was abolished on addition of equal amount of Ca^{2+} ions. Thus of the three multiple forms of PG, only PG1 activity was stimulated by Ca^{2+}. Trivalent Fe slightly inhibited PG2 and PG3 activities with no effect on PG1 activity.

Table 2. Effect of metal ions and EDTA on activities of multiple forms of polygalctouronase

Metal ions	Concentration (mM)	Residual activity (%) PG1	PG2	PG3
None	—	100	100	100
Na^+	10	118	110	140
K^-	10	127	120	120
Mn^{2+}	1	122	100	80
Mg^{2+}	1	115	95	85
Ba^{2-}	1	104	70	80
Cd^{2+}	1	82	80	60
Hg^{2-}	1	55	65	40
Pb^{2+}	1	63	45	40
Cu^{2-}	1	95	100	80
Ca^{2+}	1	150	100	100
	5	162	105	110
	10	186	110	105
EDTA	10	75	150	95
Ca^{2+} + EDTA	10 + 10	110	100	110
Fe^{3+}	1	105	90	80

All three forms of the enzyme showed activation on addition of monovalent salts. The effect of monovalent salts on polygalacturonase activity is due to salt stimulation of activity interacting with a salting out of substrate. Activation of PG activity by monovalent salts has been reported in tomato fruit [16].

Effect of detergents

PG1 activity was less sensitive to the sulfonic detergent, Teepol, compared with PG2 and PG3. In 0.1% concentration PG2 and PG3 activities were completely inhibited or inactivated, whereas PG1 activity was inhibited by 25%. The non-ionic detergent, Triton X-100, did not significantly alter the activity of any of three forms of PG. The cationic detergent, CTAB at 0.1%, strongly inhibited PG3 activity by 40%, with no effect on PG1 and PG2 activities.

Effect of sulfhydryl binding agent and sulfhydryl agent

All three multiple forms of the enzyme were inhibited by p-CMB. The sulfhydryl agent, 2-ME, did not produce any effect on PG1 activity, but activated PG2 and PG3 activities by 40 and 150%, tested at 5 mM. Activation of an enzyme preparation [22, 23] by the incorporation of sulfhydryl agents lends support to the need for sulfhydryl groups for the functioning of the enzyme. The activity of PG1 was not influenced by incorporation of sulfhydryl agent in the assay system and therefore it, does not appear to be a sulfhydryl enzyme.

M_r *determination*

M_r was determined by gel filtration on Sephacryl column. A curve between Ve/Vo and log M employing standard proteins was drawn (Fig. 3). From this plot, M_r for PG1, PG2 and PG3 were found to be 23,200, 58,000 and 130,000, respectively. Banana PG1 was distinct from the other two forms in being of small M_r. Usually such a small M_r is not reported for plant polygalacturonases. The M_r of PG2 was similar to that of apple exo-PG [10], strawberry exo-PG [14] and apple endo-PG [24]. The M_r of one of the forms of tomato PG has been reported as 42,000 [12], 43,000 [16], 44,000 [15] and 45,700 [25]. The M_r of banana PG3 was close to that of one of the forms of tomato PG, namely 115,000 [16]. A high M_r 199,500 has also been reported [25] for tomato PG.

The large M_r form of the enzyme was most prominent in the early ripening period of tomato fruit [12]. In contrast, PG3, the large M_r form of the banana enzymes, was negligible in the early ripening period and increased progressively during ripening, attaining the maximum value in the fully ripe fruit.

Reaction rate-substrate concentration relationship

Kinetic studies were carried out using polygalacturonic acid (PGA) in the range 0.03–0.35%. The response of PG1 and PG2 to increasing substrate concentrations was typically Michaelian. On the other hand, the substrate saturation curve for PG3 enzyme was biphasic in shape. The *Km* values calculated from the double reciprocal plots using the software Sigma plot, version 4.0 were 0.22 and 0.14%, respectively for PG1 and PG2. Thus PG2 showed higher affinity towards its natural substrate, PGA, than PG1.

Identification of reaction products

No detectable galacturonic acid spot on paper chromatograms appeared as the product when the enzymic reactions for PG1 and PG3 were run for up to 60 min. A distinct spot corresponding to galacturonic acid was observed in PG2 reaction mixture, the intensity of which increased with time of incubation up to 60 min.

The reaction products were further identified on the basis of viscosity measurement and determination of reducing sugar released during different time of enzyme assays. In these experiment 3% of the substrate was hydrolysed. A sharp decrease in viscosity was found in PG1 and PG3 preparations with a slow increase in reducing sugars, indicating that PG1 and PG3 are endo-polygalacturonases. In PG2, the viscosity decreased slowly with time, but reducing sugar content increased sharply, suggesting PG2 as an exo-polygalacturonase. PG2 and PG3 increased during the ripening of banana fruits. PG3, the endo-PG, is likely to catalyse the conversion of de-esterified pectin molecules to the soluble smaller fragments of pectin, which is then completely hydrolysed to D-gal-

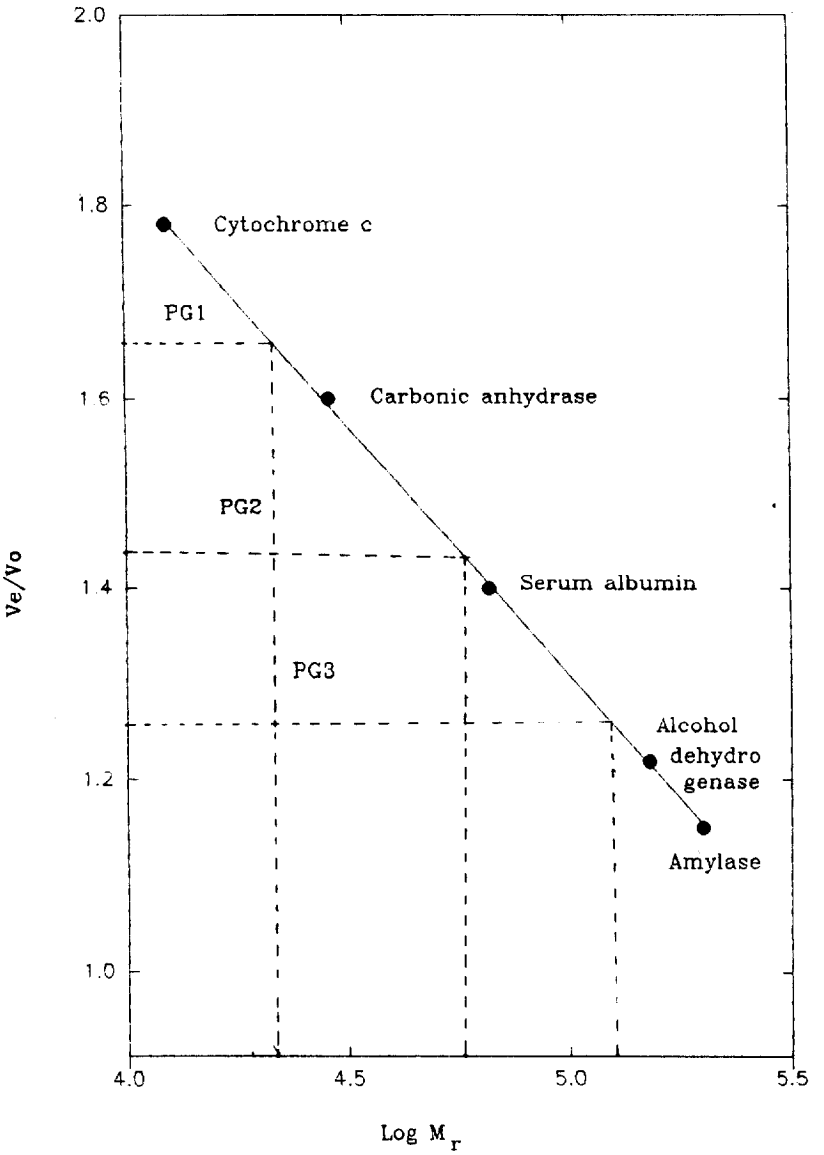

Fig. 3. Estimation of the M_r of multiple forms of polygalacturonase from ripe banana fruits on Sephacryl S-200 column (1.6 × 95 cm). The position of three forms of the enzyme are shown by broken lines.

acturonic acid by PG2, the exo-PG. Increase in monogalacturonic acid in fruit pulp during ripening of banana fruit has been reported [26]. The softening of banana fruits that accompanies ripening appears to be due to extensive degradation of pectin by the coupled action of PG3 and PG2. Endo-PG has been implicated in tissue softening because it appeared during fruit ripening in tomatoes with a corresponding increase in fruit softening [27]. Other fruits, such as peaches [28], papayas [29] and pears [19] also showed elevated activity of an endo-PG during ripening. The softening in *rin* and *nor* mutants of tomatoes that were deficient in PG activity was delayed or decreased [27]. There is ample evidence to suspect that among the cell wall polysaccharides, pectins are solubilized when the tomato fruit starts ripening [30] and the solubilization of pectin is thought to be a direct function of polygalacturonase activity [31]. Studying the effect of humidity on ripening in banana fruit, the loss in flesh firmness, which was rapid in fruits held at low RH, was positively correlated with increased polygalacturonase activity [32]. However, the extent of these events *in vivo* remains ill defined at present [33]. Polypeptides which have the characteristics of glycoproteins are reported to accumulate in ripening banana pulp [34]. One of these sets of proteins appears to be polygalacturonase, since it cross reacted with antibodies to tomato polygalacturonase. The experiments on transgenic tomatoes indicate that fruit softening is not affected significantly in tomatoes with down-regulated PG activity [35, 36] and suggest further that although PG activity causes depoly-

merization of polyuronides in fruits, the enzyme is not the sole cause of the cell wall structural changes during the softening process. Working on transgenic (*rin*) tomato fruits, it was observed that when the expression of PG gene has been up-regulated using the promoter region of another ripening associated gene, PG production and polyuronide degradation are increased without any detectable effect on fruit softening [37].

EXPERIMENTAL

Fruit tissue

Banana (*Musa acuminata* cv. Hari Chhal) fruits were collected from a field. Fingers from different stages of ripening including pre and post climacteric were selected for the experiments.

Enzyme preparation

Banana fingers were freed from peel, sliced longitudinally and the central core carrying seeds cut out and discarded. The residual pulp was cut into small pieces and processed immediately. In a typical experiment 115 g tissue was homogenized in a Waring blender with 350 ml of cold freshly prepared homogenizing medium consisting of 0.02 M Na-Pi buffer, pH 7, neutralised 0.02 M EDTA, 1% Triton X-100, 0.02M cysteine-HCl, and 1 mM PMSF. The motor was operated at top speed for 2 min with 2 intervals. The homogenate after straining through 2 folds of muslin cloth was made up to 380 ml with the medium and centrifuged at 15,000 *g* for 30 min. $(NH_4)_2SO_4$ was added to 0.9 satn. The suspension centrifuged at 20,000 *g* for 45 min and the ppt suspended in 40 ml of 0.02 M Na-Pi buffer, pH 7 containing 1 mM PMSF. The suspension was dialysed against the same buffer and centrifuged at 15,000 *g* for 15 min.

Partial purification of multiple forms of polygalacturonase

$(NH_4)_2SO_4$ fraction (50 ml = 570 mg protein) was loaded onto a (3.2 × 24 cm) DEAE-cellulose column, pre-equilibrated with 0.02 M Na-Pi buffer pH 7. The column was washed with 3 bed vol of the above buffer and 8 ml frs collected. The adsorbed proteins were eluted using a linear NaCl gradient (0–1 M). Frs 78 to 85 (0.12–0.22 M NaCl) were pooled and constituted PG2, whereas pooled frs 119 to 126 (0.74–0.84 M NaCl) constituted PG3. The unadsorbed proteins were concd with sucrose and loaded onto a CM-cellulose column (3 × 10 cm), pre-equilibrated with 0.02 M NaOAc buffer, pH 5. The adsorbed enzyme was eluted with 0.02 M Na-Pi buffer, pH 7. Pooled active frs obtained from DEAE-cellulose chromatography or by CM-cellulose chromatography were concd with sucrose and further purified by gel filtration over Sephadex G-150 column (1.6 × 80 cm), pre-equilibrated with 0.02 M Na-Pi, pH 7. Frs of 3 ml were collected. In each case a single peak of activity was obtained: PG1 (frs 50–54), PG2 (frs 33–35), PG3 (frs 21–25).

Enzyme assays

Polygalacturonase activity was assayed by measuring the formation of reducing groups by DNS method [38]. The reaction mixture contained 0.2 ml of 0.2 M HOAc buffer (pH 4.5) 0.3 ml of 1% PGA pH (4.5), an appropriate amount of enzyme preparation and H_2O in a total vol. of 1 ml. The reaction, initiated by the addition of enzyme, was continued for 60 min at 37° and terminated by addition of DNS. One unit of polygalacturonase catalysed the liberation of 1 nmol of galacturonic acid in 1 s under the conditions of the enzyme assay. Pectin methyl esterase activity was measured as described in Ref. [39].

Protein determination

Protein determination was carried out according to the method of Ref. [40], using BSA as a standard.

M_r *determination*

The M_r of multiple forms of PG was determined by gel filtration chromatography on a Sephacryl S-200-HR column by the method of Refs [41, 42], using cytochrome-c (M_r 12,400), carbonic anhydrase (M_r 29,000), albumin (M_r 66,000), alcohol dehydrogenase (M_r 150,000) and β-amylase (M_r 200,000) as reference proteins.

Identification of reaction products

1 ml of PG reaction mixture at the end of 1 h incubation was heated at 100° for 10 min to stop the reaction. The undegraded substrate was pptd by addition of 5 vol. chilled (95%) EtOH. The ppt was centrifuged for 15 min at 12,000 *g* and the supernatant lyophilised. The lyophilised sample was applied to a Whatman No. 1 chromatographic paper and developed in a solvent system *n*-BuOH–HOAc–H_2O (12:3:5) with D-galacturonic acid as a standard. The spots were visualized with aniline phthalate reagent.

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