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Multiple forms of polygalacturonase from mango (*Mangifera indica* L. cv Alphonso) fruit

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Abstract

Polygalacturonase (PG) from mango pulp revealed three isoforms (I, II, III) upon ion exchange and gel filtration chromatography, each having an abundance of 68%, 6% and 26%, and molecular weights (M_r) 40, 51 and 45 kDa, respectively. The pH optimum for the isoforms was between 3 and 4. PG-I was stable over a wide pH range (4–7.5) unlike PG II and III, which were stable at pH 4 and 5, respectively. The optimum temperature was around 40 °C for all the three isoforms. Their apparent K_m for pectic acid was in the range 0.22–0.25 mg ml^{-1}. The V_{max} for PG I, II and III was 5.7, 3.6 and 4.4 μmol GalA equivalent h^{-1}, respectively. Cd^{2+}, Cu^{2+} and Fe^{2+} and EDTA inhibited whereas GalA, Gal, Fuc, Rha and Ara stimulated PG-I activity, in particular. The major endogenous substrates for mango PG were identified to be two rhamnogalacturonans varying in their sugar ratio. These results are discussed in the light of pectin dissolution in vivo in ripening mango.

Keywords: Mango; Polygalacturonase; Pectin; Endogenous substrates; Ripening; Textural softening

1. Introduction

Pectolytic enzymes are widespread in plants, fungi and bacteria. They are industrially useful enzymes for extraction, clarification and liquefaction of fruit juices and wines, retting plant fibers and de-clogging pulps. They act on plant tissues, especially on pectins, causing cell lysis. Pectins are the main components of middle lamella and primary cell wall of plant cell. The most apparent changes during ripening of many fruits are decrease in pectin molecular weight (M_r) and increase in soluble polyuronides, which correlate with increased activity of polygalacturonase (PG) (Crookes & Grierson, 1983). PG, an important pectolytic glycanase, is the primary enzyme playing a significant role in pectin dissolution in vivo. At the cell wall level, this is of great significance leading to textural softening and loosening of cell structure (Brownleader et al., 1999). PG activity was reported in a number of ripening fruits (Lang & Dornenburg, 2000), but has been purified and studied only from very few fruits where the existence of PG isoforms was also shown (Ali & Brady, 1982; Pathak & Sanwal, 1998). PG gene was the first to be cloned for studying textural regulation in tomato, and the transformed tomato with PG antisense gene resulted in improved fruit with firmer texture and extended shelf life

Abbreviations: PG, polygalacturonase; IEC, ion exchange chromatography; DEAE, diethylaminoethyl; GPC, gel permeation chromatography; M_r, molecular weight; kDa, kilo daltons; PGA, polygalacturonic acid; GalA, galacturonic acid; Gal, galactose; Glc, glucose; Man, mannose; Fuc, fucose; Rha, rhamnose; Ara, arabinose; Xyl, xylose; EDTA, ethylenediaminetetraacetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVP, polyvinyl pyrrolidone; PMSF, phenylmethylsulfonylfluoride; rpm, revolutions per minute.

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(Hadfield & Bennett, 1998; Smith et al., 1988). This gave remarkable clues regarding the role of PG in fruit cell wall metabolism.

Despite similar catalytic properties, PGs differ from fruit to fruit, thus reducing the percent homology of PG genes. Mango, being commercially an important fruit of India, has low levels of PG, compared to tomato (Labib, El-Ashwah, Omran, & Askar, 1995; Lazan, Ali, Wah, Voon, & Chaplin, 1986) and has not been studied so far. The present investigation describes for the first time the method for purification of three distinct isoforms of PG, their properties and action pattern on endogenous substrates purified from mango (Prasanna, Prabha, & Tharanathan, 2004a).

2. Materials and methods

2.1. Plant material

Mature green mango (*Mangifera indica* L. cv. Alphonso) fruits were freshly harvested from a local farm and kept at ambient temperature for normal ripening. The four stages of ripening chosen are as follows: (I) mature unripe (dark green); (II) pre-climacteric (light green); (III) post-climacteric (yellowish green/intermediate); and (IV) ripe (yellow, soft). Acetone dried powder from mango, at post-climacteric stage III of ripening was prepared (Chan & Tam, 1982) and used for enzyme isolation and purification. All the determinations were done in triplicate and the mean value was represented.

2.2. Extraction and purification of PG from mango

All extraction and purification steps were performed at temperature not exceeding 5 °C. The acetone-dried powder (20 g) of mango pulp was homogenized with the extraction buffer (150 ml) in a Sorval mixer and incubated overnight at 4 °C (Prasanna, Prabha, & Tharanathan, 2004b). The extraction buffer consisted of 0.1 M citrate buffer containing high ionic strength salt (1.3 M NaCl), 13 mM EDTA, 1% PVP, 0.1% PMSF and 0.1% cystenium HCl. The resultant slurry was filtered through cheese cloth and clarified by centrifugation at 7000 rpm for 15 min. The crude enzyme extract, dialyzed and concentrated using sucrose solution, was applied onto DEAE-cellulose column (12.5 × 3.2 cm) and eluted first with 20 mM acetate buffer (pH 4.8) and then with the same buffer containing linear gradient of NaCl (0–1 M). Fractions (4 ml) were collected and the enzyme active fractions were pooled separately, dialyzed, concentrated, and applied onto a column of pre-calibrated Sephadex G-200 (85 × 1.2 cm), equilibrated with 50 mM acetate buffer (pH 4.8) containing 0.05 M NaCl. Fractions (2.5 ml) were collected and the M_r of the PG isoforms was calculated using marker proteins, such as cytochrome *C* (12 kDa), ovalbumin (44 kDa) and bovine serum albumin (66 kDa). The active fractions were pooled, dialyzed, concentrated, assayed for protein and enzyme activity, and used for further studies.

2.3. Enzyme assay

Due to low activity of mango PGs, 2 h incubation time was used throughout, unless otherwise mentioned. The reaction course was nearly linear, as reported (Labib et al., 1995). A suitably diluted enzyme solution was added to a reaction mixture containing 0.1 mM acetate buffer (pH 3.8) and 2.5 mg ml^{-1} polygalacturonic acid (PGA, Sigma Chemical Co., USA) and adjusted to pH 4.0 with 1 N NaOH, in a total volume of 1 ml. The reaction was incubated at 37 °C for 2 h and later terminated by adding potassium ferricyanide reagent (2 ml), followed by heating at 100 °C for 20 min (Imoto & Yagishita, 1971). The reducing sugar released was measured (as GalA) spectrophotometrically at 420 nm. One unit of enzyme activity was defined as number of μmol reducing sugar equivalent to GalA released per hour under standard assay conditions (1 unit = 0.275 nKat).

2.4. Protein estimation

Protein was estimated by modified Bradford's method, using bovine serum albumin as standard (Zor & Selinger, 1996).

2.5. Electrophoresis

SDS–PAGE was performed on a 10% (w/v) polyacrylamide gel, by the method of Laemmeli (1970), using suitable marker protein standards (Pharmacia, Sweden). Gels were stained for protein with silver nitrate (Porro, Viti, Antoni, & Saletti, 1982).

2.6. Enzymic properties of mango PG isoforms

The effect of pH on the activity of PG isoforms towards PGA was examined at 37 °C over a wide range of pH in 100 mM of buffers (KCl:HCl buffer, pH 1.2–2.0; glycine:HCl buffer, pH 2.2–3.4; acetate buffer, pH 3.6–5.4; phosphate buffer, pH 5.8–8.0; glycine:NaOH buffer, pH 8.4–10.0). Suitably diluted purified enzyme was added to the reaction mixture containing buffer at defined pH and 2.5 mg ml^{-1} PGA, incubated for 2 h at 37 °C and estimated the reducing group released (as GalA). The effect of pH on the stability of PG isoforms was determined by incubating the enzyme in 100 mM buffer at different indicated pHs (as mentioned above) for 24 h at 4 °C. The residual activity of the enzyme was determined after adjusting to optimum pH.

The effect of temperature on the hydrolytic activity of PG isoforms towards PGA at optimal pH was examined

at different temperatures between 27 and 77 °C. The purified enzyme was incubated for 2 h with 100 mM acetate buffer containing 2.5 mg ml^{-1} PGA, after which the reducing group released, was estimated. The stability of PG isoforms at different temperatures was determined by incubating purified enzyme with 100 mM acetate buffer (optimum pH) for 15 min at different temperatures in the range 27–77 °C. The residual activity was immediately assayed. T_m was the temperature at which 50% of the activity was retained.

The kinetic parameters were determined by incubating suitably diluted purified enzymes with 100 mM acetate buffer (at optimum pH) containing PGA 0.1–2.5 mg ml^{-1} at 37 °C for 2 h. The Michaelis–Menten constants, K_m and V_{max}, for substrate hydrolysis were calculated by double reciprocal Lineweaver–Burk plot.

The effect of metal ions (1 mM) and structural analogues (2 mM) on the hydrolytic activity of PG isoforms was determined by assaying the residual activity, after incubating the enzymes in 100 mM acetate buffer (optimum pH) containing the respective metal ion or structural analogue, for 20 min at 4 °C.

The hydrolytic activity of PG isoforms towards different substrates at optimum pH was examined by incubating at 37 °C for 2 h in 100 mM acetate buffer containing PGA (control, 0.6 mg ml^{-1}), citrus pectin (0.6 mg ml^{-1}), galactomannan (0.45 mg ml^{-1}), and carboxymethyl cellulose (0.45 mg ml^{-1}), after which the reducing group released was estimated. For endogenous activity experiments, each of the three purified substrates; arabinogalactan (1 mg) and rhamnogalacturonans (0.5 mg, each), extracted, fractionated, and GPC purified from unripe mango pulp (Prasanna et al., 2004a; Prasanna, Yashoda, Prabha, & Tharanathan, 2003), was incubated with the PG isoforms in a total volume of 0.5 ml at 37 °C for 7 h. The incubation time was increased to get measurable amount of reducing sugar from the purified substrates. Controls containing only substrates were also incubated simultaneously. The reaction was stopped by heating at 100 °C for 3 min and assayed for reducing sugar.

3. Results and discussion

3.1. Purification of mango PG

The total PG activity, when monitored at different stages of ripening showed an increased activity reaching a maximum at the post-climacteric stage of ripening (stage-III) (Fig. 1). This also indicated that there is a gradual increase in the PG protein expression during ripening, as reported for other fruits (Brownleader et al., 1999; Lang & Dornenburg, 2000). A similar increase in activity during ripening was reported in other fruits (Brownleader et al., 1999; Crookes & Grierson, 1983; Pathak & Sanwal, 1998). The citrate:NaCl buffer extract of acetone dried powder of mango fruit pulp (stage-III) showed high specific activity (data not shown), and was used for further purification of mango PG. As the extraction was carried out with acetone-dried powders, the amount of extracted protein was low. The crude mango PG, extracted at 4 °C was reported to be stable even up to 25 °C (Labib et al., 1995).

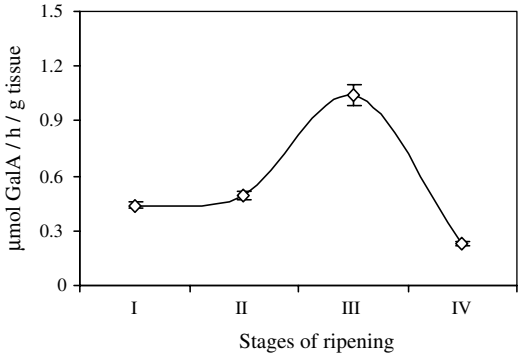

Fig. 1. Activity of polygalacturonase at various stages during ripening. Scattered bars indicate deviation from the mean value. I. Mature unripe (dark green); II. pre-climacteric (light green); III. post-climacteric (yellowish green/intermediate); IV. ripe (yellow, soft). (1 µmol GalA equivalent released h^{-1} (1 Unit) = 0.275 nKat). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Ion exchange chromatography (IEC) on DEAE-cellulose resolved PG into PG-I, -II and -III, with relative abundance of 68%, 6% and 26%, respectively (Fig. 2). PG-I (the most abundant isoform) eluted in the buffer wash, before gradient elution. The unadsorbed PG-I was not retained even on CM-cellulose column. The bound PG activity upon elution with linear gradients of NaCl was eluted between 0.1–0.2 M (PG-II) and 0.2–0.35 M (PG-III). All the three isoforms were purified by GPC on Sephadex G-200, which showed M_r in the range of 40–51 kDa (Fig. 3). Native as well as SDS–PAGE revealed single protein bands for these three isoforms, confirming, thus their homogeneity. The M_r of mango PG isoforms (~40–51 kDa, Fig. 4) was slightly lower when compared to other fruit PG isoforms, which were in the range of 50–59 kDa, except that of banana PG-I and -III which showed very low (23.2 kDa) and very high (130 kDa) molecular weight values (Nogata, Ohta, & Voragen, 1993; Pathak & Sanwal, 1998; Pressey & Avants, 1975; Smith et al., 1988). The later (high molecular weight banana PG-III) was reported to be a glycoprotein, and made up of small subunits of 30 kDa (Pathak, Mishra, & Sanwal, 2000).

3.2. Characterization of mango PG

Table 1 summarizes the purification characteristics of PG from mango pulp. The total specific activity increased from 1.8 to 12 U mg^{-1} (0.5–3.3 nKat mg^{-1})

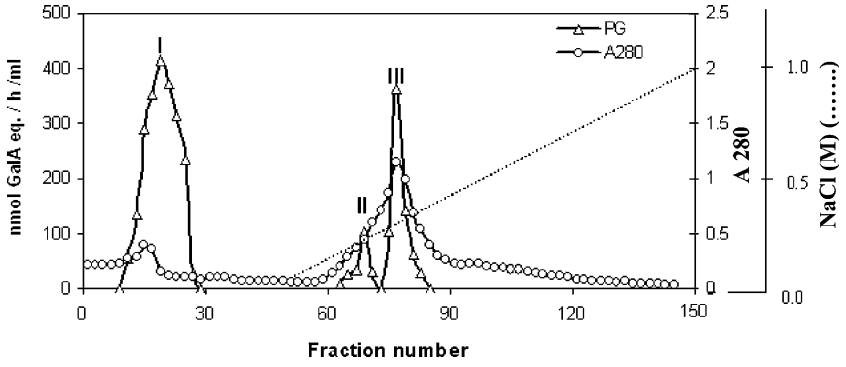

Fig. 2. IEC profile of PG isoforms on DEAE-cellulose. The experimental conditions used are described in Section 2. I, II and III indicate PG isoforms I, II and III, respectively.

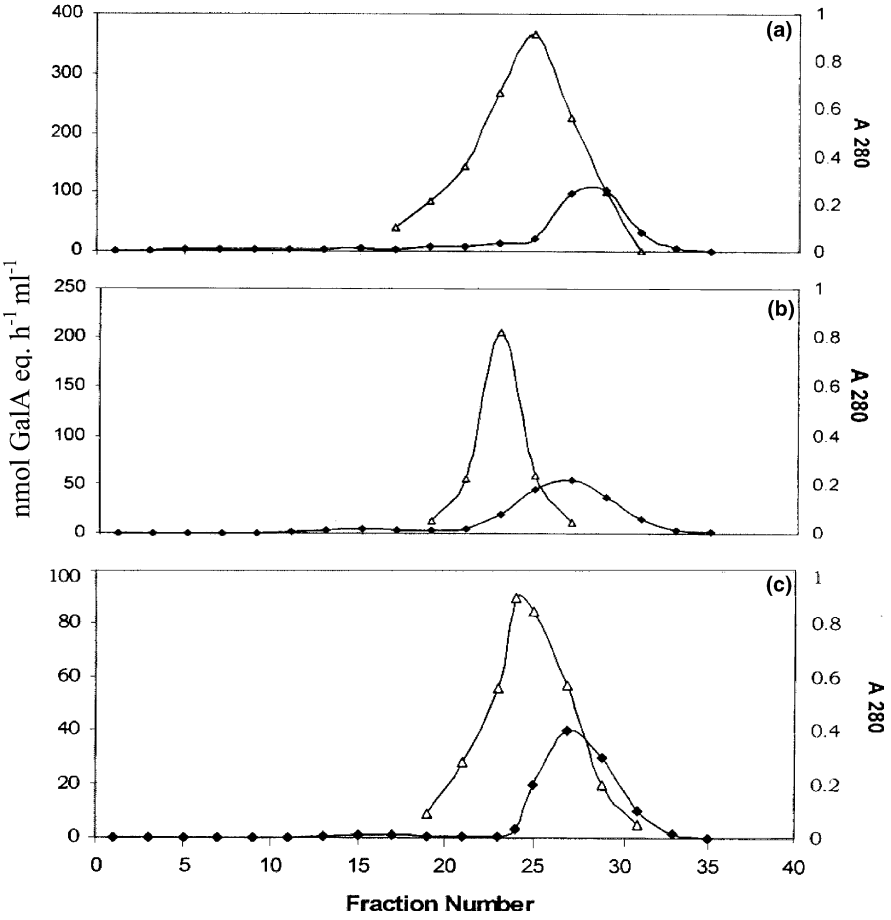

Fig. 3. GPC profile of PG isoforms on Sephadex G-200. The experimental conditions used are described in Section 2: (a) PG-I, (b) PG-II, (c) PG-III.

upon IEC purification, which increased further to 93 U mg^{-1} (25.6 nKat mg^{-1}) upon GPC. The specific activity for PG-I, -II and -III was 3.8, 3 and 5 upon IEC, while it was 34, 30 and 29, respectively,upon GPC. PG-I was less stable than the other two isoforms.

Though, the total PG activity in mango fruit is much less when compared to tomato (Lazan et al., 1986), banana and papaya (unpublished data), mango PG was expressed as three distinct isoforms at the post-climacteric stage, as in the case of banana and strawberry fruit PGs (Nogata et al., 1993; Pathak & Sanwal, 1998). Peach and Pear fruit showed two isoforms of PG (Pressey & Avants, 1973a, 1973b). In tomato, differential expression of PG isoforms at different stages of ripening was demonstrated (Crookes & Grierson, 1983; Pressey, 1987; Pressey & Avants, 1973c).

The enzymic properties were studied with GPC purified PG isoforms. The pH optima was found to be 3.2,

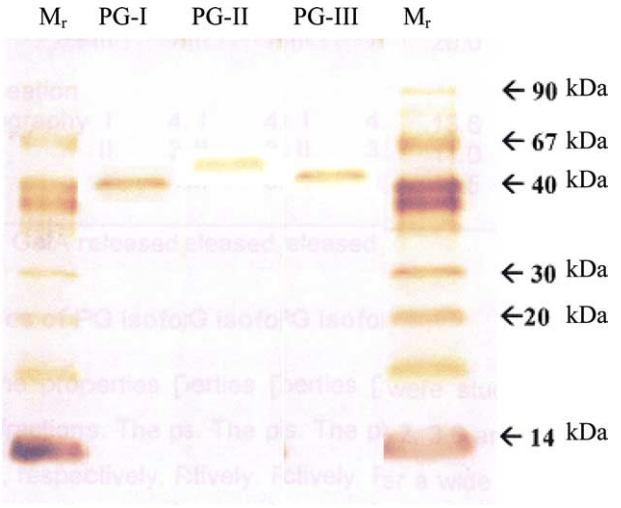

Fig. 4. SDS–PAGE of purified PG-I, PG-II and PG-III. Left and right lanes: reference M_r marker proteins in the range 14–94 kDa.

Table 1
Purification of PG from mango

Fractions	Total activity* (U)	Protein (mg)	Sp. activity (U mg^{-1})	Yield (%)	Fold purification
Crude	36.47	20.6	1.77	100	1.00
Anion exchange chromatography					
PG-I	8.87	2.30	3.86	24.3	2.2
PG-II	5.17	1.73	2.99	14.2	1.7
PG-III	7.31	1.40	5.22	20.0	3.0
Gel permeation chromatography					
PG-I	4.95	0.14	34.36	13.6	19.4
PG-II	3.00	0.10	30.00	11.0	17.0
PG-III	3.82	0.13	29.38	10.5	16.6

U = μmol GalA equivalent released h^{-1} (1 U = 0.275 nKat).

3.6 and 3.9 for PG-I, -II and -III, respectively (Fig. 5(a)). The PG isoforms from mango resembled those of banana fruit in their pH optima, which was ~3.5, while those of cucumber, peach, pear, tomato and strawberry showed a higher pH optima in the range of 4–5.5 (Chan & Tam, 1982; Nogata et al., 1993; Pathak & Sanwal, 1998; Pathak et al., 2000; Pressey, 1987; Pressey & Avants, 1975, 1973a, 1973b). PG-I showed a pH stability over a wide range of pH 4.0–7.5, while PG-II and -III showed pH stability at pH 4.0 and 5.0, respectively (Fig. 5(b)).

The temperature optima were found to be 37, 42 and 37 °C, respectively, for PG-I, -II and -III (Fig. 5(c)). Temperature optimum for crude mango PG was reported to be between 30 and 35 °C (Labib et al., 1995), while purified banana PG-III showed maximum activity at 40 °C (Pathak et al., 2000). As crude mango PG is heat-labile (Labib et al., 1995), PG isoforms were pre-incubated at different temperatures ranging from 27 to 77 °C for 15 min only, after which the remaining enzyme activity was assayed. Even at 37 °C, 30–35% of the original activity of PG-I and PG-II was lost after 15 min of incubation (Fig. 5(d)). PG-III appeared to be more thermostable (T_m = 69 °C) than PG-I and -II. The stability of crude mango PG was determined after incubation at various temperatures for 20 min only (Labib et al., 1995). Experiments with higher incubation time (18 h) for activity and lower incubation time (5 min) for thermal stability were reported for PGs from strawberry and banana fruits (Nogata et al., 1993; Pathak & Sanwal, 1998).

The apparent K_m and V_{max} values for PG-I, -II and -III were 0.25, 0.23 and 0.22 mg ml^{-1}, and 5.7, 3.6 and 4.4 μmol GalA equivalent released h^{-1}, respectively (Fig. 5(e)). The low K_m values indicate that mango PGs have high affinity towards the PGA.

As for the effect of metal ions and EDTA, at 1mM level (Table 2), Cd^{2+} showed a percent inhibition of 76, 57 and 100, respectively, for PG-I, -II and -III. Fe^{2+} and Cu^{2+} inhibited only PG-III to the extent of 55%. EDTA showed 100% inhibition for PG-III, indicating the strong requirement for the metal ion for activity. Considerable stimulation of PG-II activity by Ca^{2+} and Zn^{2+} was observed. Zn^{2+} showed slight activation of PG-I and III, and Mg^{2+} showed slight activation of PG-I only. The major metal ion inhibitors for mango PGs generally were Cd^{2+}, Fe^{2+} and Cu^{2+}. Cd^{2+} was shown to be slightly inhibitory to tomato exo-PG (Pressey, 1987). The inhibition by Cu^{2+} may be due to the oxidation of sulfydril groups, indicating that the sulfydril groups play an important role in activity. Similar inhibition was reported for banana PG (Pathak & Sanwal, 1998). Additionally, unlike in mango, divalent ions like Mn^{2+}, Mg^{2+}, Pb^{2+} and Hg^{2+} inhibited banana PG (Pathak & Sanwal, 1998). However, Mg^{2+} showed stimulation of PG-I activity in mango. The inhibition of mango PG by EDTA was similar to that of cucumber and strawberry PGs (Pressey & Avants, 1975; Nogata et al., 1993). Activation of PG-II of mango by Ca^{2+} was also similar to Ca^{2+} activation of PG-I of banana (Pathak & Sanwal, 1998), and exo-PG of tomato (Pressey, 1987). These results indicate that divalent metal ions like Ca^{2+}, Zn^{2+} and/or Mg^{2+} stimulate PG activity probably by stabilizing the negatively charged carboxyl groups on pectins, and their removal by EDTA-chelation decrease or inhibit PG activity. Interestingly, EDTA activated PG-II of banana fruit (Pathak & Sanwal, 1998).

The effect of product analogues (monosaccharides) on PG isoforms is depicted in Table 3. All the sugars tested, except Fuc, showed inhibition on PG-II. Man and Xyl inhibited PG-I and -III, considerably, while Glc inhibited PG-I and PG-II. Strikingly, PG-II was stimulated by sugars like GalA, Gal, Fuc, Rha and Ara, while PG-III was activated only by GalA and Fuc, and by Glc to some extent. It is worth noticing that PG-II was not activated by any sugars tested, instead it

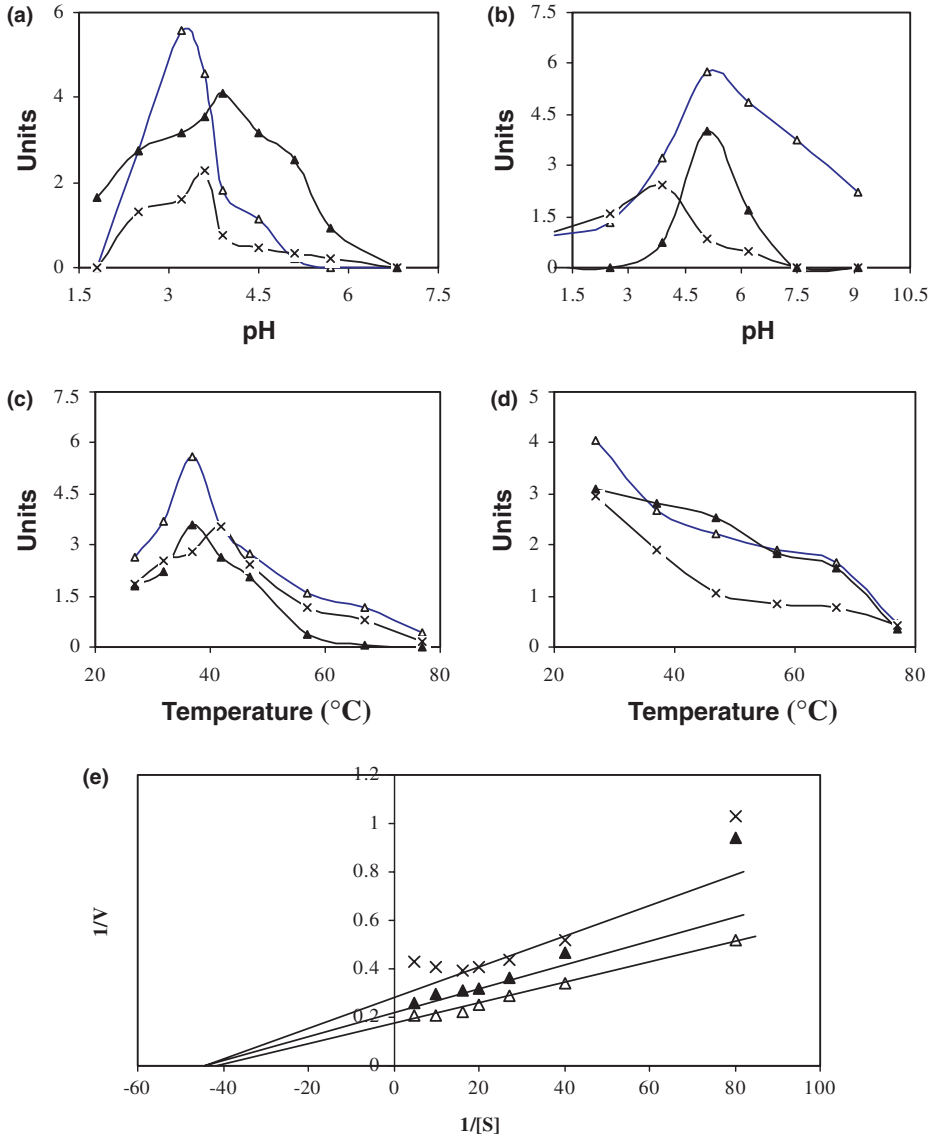

Fig. 5. Properties of mango PG isoforms. Values represent the mean of three independent Experiments (triplicate): (a) pH optimum; (b) pH stability; (c) temperature optimum; (d) temperature stability; (e) double reciprocal Lineweaver–Burk plot (-△- PG-I; -×- PG-II; -▲- PG-III) Units [V] = μmol GalA equivalent released h^{-1} (1 Unit = 0.275 nKat), [S] = 0.1–2.5 mg ml^{-1} PGA.

Table 2
Effect of metal ions and EDTA on activity of PG isoforms of mango

Metal ions (1 mM conc.)	% Activity over control		
	PG-I	PG-II	PG-III
Control	100	100	100
Ca^{2+}	100	156	108
Mg^{2+}	124	94	100
Fe^{2+}	95	63	46
Zn^{2+}	119	169	108
Cu^{2+}	105	63	46
Cd^{2+}	24	43	0
EDTA	86	81	0

Table 3
Effect of product analogues on the activity of PG isoforms of mango

Product analogues (2 mM conc.)	% Activity over control		
	PG-I	PG-II	PG-III
Control	100	100	100
GalA	114	75	140
Gal	133	18	60
Glc	29	36	116
Man	10	4	40
Fuc	276	100	216
Rha	295	71	96
Ara	176	18	36
Xyl	19	23	12

was inhibited. Thus, the degradation products of cell wall degrading enzymes may inhibit/activate the PG enzyme activities to some extent.

There was considerable hydrolytic activity by all the three PGs on galactomannan and purified endogenous substrates (Table 4). PG-I and -II showed little hydro-

Table 4
Activity of PG isoforms of mango on natural and endogenous substrates

Substrates	% Activity over control		
	PG-I	PG-II	PG-III
Control (0.06% PGA)	100	100	100
Natural substrates			
Citrus pectin (0.06%)	0	0	0
Galactomannan (0.045%)	38	29	20
CM-cellulose (0.045%)	0	0	0
Endogenous substrates			
Arabinogalactan	8	9	0
Rhamnogalacturonan 1	57	73	75
Rhamnogalacturonan 2	43	55	61

lytic activity towards arabinogalactan, while PG-III showed no activity. When assessed for in situ hydrolysis and endogenously hydrolysable substrates in mango pulp, the two major pectic polymers, i.e., two rhamnogalacturonans, were found to be susceptible (Table 4). The latter were composed of GalA, Ara, Gal and Rha in the relative ratio of 69:15:13:2 and 62:10:23:4, respectively (Prasanna et al., 2004a; Prasanna et al., 2003). Probably such an event contributes to the loosening of cell structure at the middle lamella of the cell wall. A few other enzymes such as galactanase, arabinanase and β-galactosidase were also found to be very active in mango (Bhagylakshmi et al., 2002; Prasanna et al., 2004b), which collectively may be responsible for total pectin solubilization in vivo by hydrolysing the arabinogalactan side chains.

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