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Purification and characterisation of multiple forms of polygalacturonase from mango (*Mangifera indica* cv. Dashehari) fruit

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ABSTRACT

Three multiple forms of polygalacturonase (PG) namely PGI, PGII and PGIII were isolated, purified and characterized from ripe mango (*Mangifera indica* cv. Dashehari) fruit. Native molecular weights of PGI, PGII and PGIII were found to be 120, 105 and 65 kDa, respectively. On SDS–PAGE analysis, PGI was found to be a homodimer of subunit size 60 kDa each while those of PGII and PGIII were found to be heterodimers of 70, 35 and 38, 27 kDa subunit size each, respectively. Three isoforms of PG differed with respect to the effect of pH, metals, reducing agents and their susceptibility towards heat. PG isoforms also differed with respect to the effect of substrate concentration on enzyme activity. PGI and PGIII exhibited inhibition at high substrate concentration while PGII did not. Km for polygalacturonic acid was found to be 0.02% for PGI.

1. Introduction

Fruit ripening is a complex metabolic event that involves changes in fruit softening and texture, and includes a general loosening and increased hydration of the cell wall, the disassembly of cell wall polysaccharides, reductions in cell-to-cell adhesion and a decline in turgor pressure (Brummell & Harpster, 2001). Polygalacturonase (PG) catalyzes the hydrolytic cleavage of α $(1 \rightarrow 4)$ galacturonan linkages of pectin which are the main constituents of middle lamella and primary cell wall of plant cells. At the cell wall level, PG is of significance leading to textural softening and loosening of cell structure (Brownleader et al., 1999). Polygalacturonases (PG) fall into the group of enzymes termed polysaccharide lyases or polysaccharide eliminases. PGs are present in various fruit essentially during ripening (Lang & Dornenburg, 2000). In addition, PG is also found in fungi, yeast and bacteria. PGs have also been reported from tomatoes (Ali & Brady, 1982; Crookes & Grierson, 1983; Pressey & Avants, 1973b) peach (Pressey & Avants, 1973a), avocado (Wakabayashi & Huber, 2001) and pears (Pressey & Avants, 1976).

Mango is a commercially important fruit of India. Various biochemical and physiological studies related to ripening have been carried out in mango (Ali, Armugam, & Lazan, 1995; Ali, Chin, & Lazan, 2004; Krishnamurthy, Patwardhan, & Subramanyam, 1971; Lazan, Ali, Wah, Voon, & Chaplin, 1986; Muda, Seymour, Errington, & Tucker, 1995; Prasanna, Prabha, & Tharanathan, 2006; Singh, Singh, Pathak, Singh, & Dwivedi, 2007; Tandon & Kalra, 1983). Multiple forms of PGs have been demonstrated in strawberry, (Nogata, Ohta, & Voragen, 1993), tomato (Ali & Brady, 1982; Crookes & Grierson, 1983; Pressey & Avants, 1973b), peach (Pressey & Avants, 1973a), pears (Pressey & Avants, 1976), avocado (Wakabayashi & Huber, 2001) and banana (Pathak & Sanwal, 1998). Recently three multiple forms of polygalacturonase have been reported from Alphonso cultivar of mango (Prasanna et al., 2006). In view of varietal variations in ripening associated changes in different cultivars, it is important to study the ripening behavior in different varieties (cultivars) of mango. In this very context, in the present study, we have taken one of the best mango variety of India namely, 'Dashehari', for studying ripening associated changes in PG. Need of studying PG in this mango variety is pertinent as this is one of the major enzymes involved in pectin catabolism in fruits during ripening. Softening in mango has been reported to be accompanied by a decline in pectin (both water and alkaline soluble) (Roe & Bruemner, 1981). Furthermore, this decline in alkaline soluble pectin was found to be correlated with the loss of firmness of the mango fruit and was also closely correlated with the increase in PG activity during fruit ripening.

2. Materials and methods

Mature mango (*Mangifera indica* cv. Dashehari) fruits, free from disease, were collected from Central Institute of Subtropical Horticulture (CISH) orchard, Lucknow (India). Harvested mature

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and unripe fruits of Dashehari mango were washed with distilled water, air dried and allowed to ripe at room temperature (30 ± 1 °C). The zero day stage of ripening corresponds to the day of harvesting of mature and unripe fruits from orchard. For enzyme isolation and purification, pulp from ripe fruits was taken and stored at −80 °C. The frozen tissues were used whenever needed.

2.1. Enzyme extraction and purification

All extraction and purification steps were performed at temperature not exceeding 5 °C. A 30% homogenate was prepared with frozen tissue in extraction buffer and incubated for 20 min at 4 °C. Extraction buffer consisted of 50 mM sodium phosphate buffer (pH 7.0), 20 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 20 mM cysteine, 0.5% Triton X-100, 0.1% β-mercapto ethanol. During homogenization 33 mg PVPP (insoluble) was added per gram of tissue. Homogenate was centrifuged at 12,000 rpm (17,212*g*) in Sorvall RC5C for 25 min at 4 °C. Supernatant was collected after passing through the muslin cloth. Clear supernatant was subjected to 30–90% ammonium sulphate saturation. The suspension was centrifuged for 30 min in Sorvall RC5C at 12,000 rpm (17,212*g*). The pellet was suspended in 20 mM sodium phosphate buffer (pH 7.0) and dialyzed against same buffer for overnight. Dialyzed ammonium sulphate fraction was loaded on diethylaminoethyl (DEAE) cellulose column pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The column was washed with 3 column volume of the above buffer and the adsorbed protein was eluted using step gradient of NaCl (0.1–1 M). The unadsorbed fractions from DEAE cellulose column was concentrated by dialysis against solid sucrose and loaded onto the carboxymethyl (CM) cellulose column equilibrated with 20 mM acetate buffer (pH 6.0). The adsorbed enzyme on DEAE cellulose and CM cellulose column was eluted with step gradient of NaCl (0.1–1 M) in 20 mM sodium phosphate buffer (pH 7.0). Active fractions obtained after the ion exchange chromatography were pooled separately and concentrated by dialysis against solid sucrose. The concentrated fractions were further purified on gel filtration chromatography using Sephadex G -200 pre-equilibrated and eluted with 100 mM sodium acetate buffer (pH 6.0).

2.2. Enzyme assay

The enzyme assay was done as described by Gross (1982) with slight modification. Reaction system (0.4 ml total volume) containing 37.5 mM sodium acetate (pH 3.5–6.0) or sodium phosphate (pH 6.5–7.5) buffer, 0.2% polygalacturonic acid (PGA) and 50–100 μl of enzyme was incubated at 37 °C for 90 min. Reactions were terminated with 1.0 ml of cold 100 mM borate buffer (pH 9.0), then 0.4 ml of 1% 2-cyanoacetamide was added, and the samples were mixed and immersed in boiling water bath for 10 min. After equilibration to room temperature, the absorbance at 276 nm was measured. Polygalacturonase activity was expressed in terms of μ katal units. One katal of polygalacturonase catalyzed the formation of 1 mole of galacturonic acid per second under the conditions of the enzyme assay.

2.3. Characterization of purified isoforms of polygalacturonase

2.3.1. Effect of pH

Effect of pH on the activity of PG isoforms was examined at 37 °C over a wide range of pH in 37.5 mM buffers (sodium acetate buffer, pH 3.5–6.0, sodium phosphate buffer pH 6.5–7.5). Suitable amount of purified enzyme was used to measure the activity.

2.3.2. Effect of temperature

Effect of temperature on the activity of PG isoforms, at optimal pH was examined at different temperatures between 30 and 70 °C. The stability of PG isoforms at different temperatures was determined by incubating purified enzyme (at optimum pH) for 5 min at different temperatures (50–70 °C).

2.3.3. Effect of polygalacturonic acid on activity of PG isoforms

Effect of polygalacturonic acid (PGA) concentrations on the activity of PG isoforms was investigated by varying PGA concentrations (0.02–0.75%) at optimal pH and temperature. K_m and V_{max} were calculated by double reciprocal Lineweaver–Burk plot.

2.3.4. Molecular weight determination of PG isoforms

Native molecular weight of multiple forms of PG was determined by gel filtration chromatography on a Sephadex G- 200 column. Cytochrome-c (Mr 12,400), albumin (Mr 66,000), alcohol dehydrogenase (Mr 150,000) and catalase (Mr 250,000) were used as reference protein.

2.3.5. Protein determination

Protein was determined by method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Native and SDS polyacrylamide gel electrophoresis

Native and SDS–PAGE of the purified isoforms was run over 10% polyacrylamide gel as described by Davis (1964) and Laemmli (1970), respectively. Subunit molecular weight of PG isoforms was determined using standard molecular weight markers in SDS–PAGE.

3. Results

3.1. Developmental profile of polygalacturonase during mango fruit ripening

Polygalacturonase activity was investigated during mango fruit ripening. PG activity did not increase much during initial 4 days of ripening. However, after day 4 it increased rapidly attaining a peak on day 8 and thereafter declined gradually (Fig. 1). During ripening, color of fruit changed gradually from green to yellow. For isolation and purification, the enzyme was isolated on day 8 of ripening.

3.2. Purification of polygalacturonase isoforms

Multiple forms of PG were purified from ripe mango pulp through successive steps of ammonium sulphate fractionations,

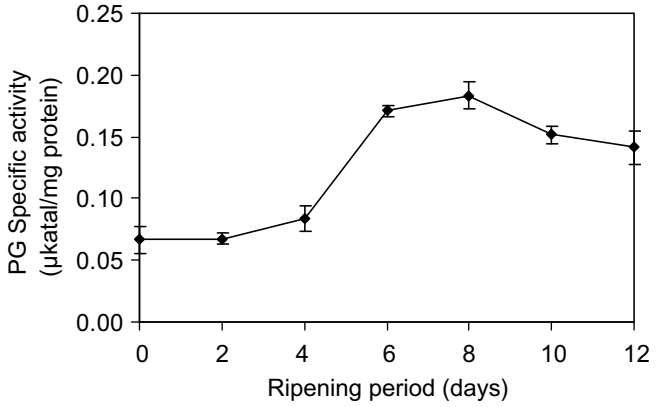

Fig. 1. Developmental profile of PG activity during mango fruit ripening at room temperature (30 ± 1 °C). The 0 day stage of ripening corresponds to the day of harvesting of mature and unripe fruits from orchard. Each value represents a mean ± SD of three independent experiments, each of them in triplicate.

ion exchange and molecular exclusion chromatography. Results are given in Table 1. Two isoforms, PGII and PGIII were bound to DEAE cellulose column while PGI did not. Isoforms PGII and PGIII were eluted with 100 mM and 500 mM NaCl, respectively. Both PGII and PGIII isoforms were further purified by gel filtration chromatography on Sephadex G-200. PGII and PGIII were purified to 22 and 26 fold with 7.5 and 11.35% recovery, respectively. Specific activity of purified PGII and PGIII were 4.15 and 4.75, respectively. The unadsorbed PGI from DEAE cellulose column was subjected to CM cellulose chromatography and eluted with 500 mM NaCl. PGI was further purified by gel filtration chromatography on Sephadex G-200 to 22 fold with a recovery of 11%. Specific activity of purified PGI was 4. Homogeneity of the purified isoforms of PG was checked by running native PAGE. A single band was observed in each case (data not shown).

3.3. Molecular weight and subunit structure

Native molecular weights of isoforms of PG were determined by gel filtration on Sephdex G-200 column and were found to be 120 kDa, 105 kDa and 65 kDa, for PGI, PGII and PGIII, respectively (Fig. 2). All the three isoforms of PG were found to be dimers. On SDS–PAGE analysis, PGI was found to be homodimer of subunit size 60 kDa each while those of PGII and PGIII were found to be heterodimer of 70 and 35 kDa and 38 and 27 kDa subunit size each, respectively (Fig. 3).

3.4. Effect of pH

Effect of pH on the activity PGI, PGII and PGIII was investigated. PGI exhibited a pH optimum at 5.0 while PGII and PGIII exhibited optimum at pH 6.0. All the isoforms showed appreciable activity in the range of pH 5–7.5 while almost negligible activity at pH 3.5.

3.5. Effect of temperature

The effect of temperature on the activity of isoforms of PG was investigated. PGI and PGII exhibited temperature optima of 37 °C while PGIII of 40 °C. The three multiple forms of the enzyme showed different heat stability. The PGIII was most heat stable, retaining 60 and 50% activity at 60 and 70 °C within 5 min, respectively. PGII was heat labile with total loss of activity at 60 °C within 5 min. PGI retained 35% activity at 60 °C, with total loss of activity at 70 °C within 5 min.

Table 1
Purification of multiple forms of polygalacturonase from post climacteric 'Dashehari' mango fruit

Steps	Total activity (μ katal)	Total protein (mg)	Specific activity (μ katal /mg protein)	Recovery (%)	Fold purification
1. Crude	33.3	180.5	0.184	–	
2. Ammonium sulphate (30–90%)	31.48	130.5	0.24	95	1.3
3. *DEAE cellulose chromatography*					
PGI (Pass through unbound)	13.8	90	0.15	41	–
PGII	4.4	15.6	0.28	13.33	1.5
PGIII	5.09	9.8	0.52	15.25	3
4. *CM cellulose chromatography*					
PGI	6.15	10.5	0.58	17.5	3.17
5. *Sephadex G-200 chromatography*					
PGI	3.6	0.9	4	10.66	22
PGII	2.5	0.6	4.15	7.5	22
PGIII	3.8	0.8	4.75	11.35	26

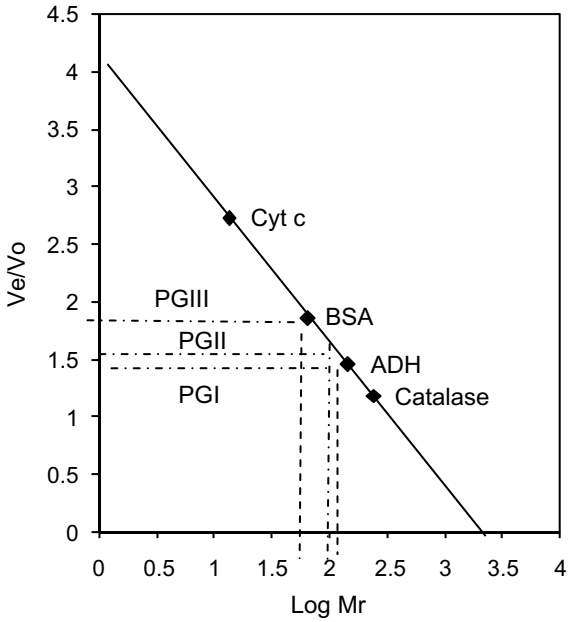

Fig. 2. Estimation of native molecular weights of multiple forms of polygalacturonase from ripe mango fruit on sephadex G-200. The position of three forms of the enzyme was shown by broken lines. Alcohol dehydrogenase (ADH), bovine serum albumin (BSA), Cytochrome-c (Cyt c) and catalase were used as standard molecular weight markers.

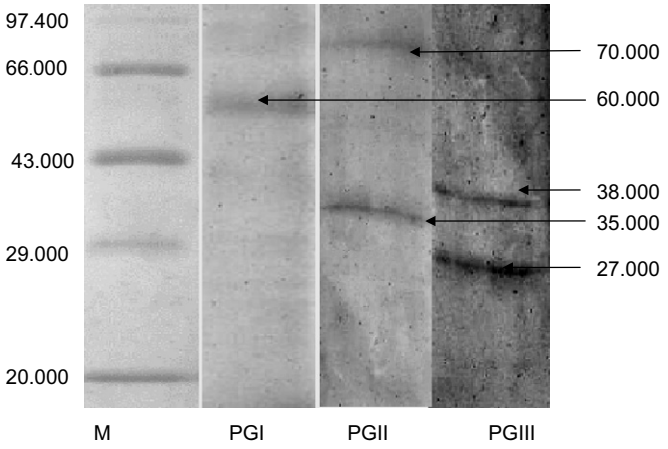

Fig. 3. SDS–PAGE analysis of purified isoforms of polygalacturonase. 10% polyacrylamide gel was run and protein staining was done using coomassie brilliant blue dye. 10 μg protein was loaded in each well.

3.6. Effect of metals

As metallic salts have been reported to influence mango fruit ripening, we have investigated the effect of various metal ions on the activity of multiple forms of PG. The multiple forms of PG showed different response to metal ions (Table 2). Monovalent metal ions have little effect on activity of polygalacturonase as compared to divalent or trivalent ones. Monovalent Na^+ and K^+ slightly stimulated PGII and inhibited PGI and PGIII. Monovalent Li^+ inhibited PGII and slightly stimulated PGI while had no effect on PGIII. Divalent Ca^{+2} activated the PGI and PGII while inhibited the PGIII. Divalent Mg^{+2} inhibited PGI and PGIII and had no effect on PGII. Trivalent Fe^{+3} is most potent activator of PGI and PGII and slightly for PGIII. EDTA was found to activate all the isoforms of PG.

Table 2
Effect of EDTA, metals and reducing agents on the activity of mango PG isoforms

EDTA/metals/reducing agents	Salt concentration (mM)	% Activity of respective control		
		PGI	PGII	PGIII
Control	–	100	100	100
EDTA	10	125	123	118
NaCl	10	92	121	95
KCl	10	94	113	100
LiCl	2	129	94	102
$CaCl_2$	10	134	150	83
$MgCl_2$	2	95	100	92
$FeCl_3$	2	593	694	136
βME	2	127	114	107
DTT	2	215	128	265
Cysteine	2	209	126	123

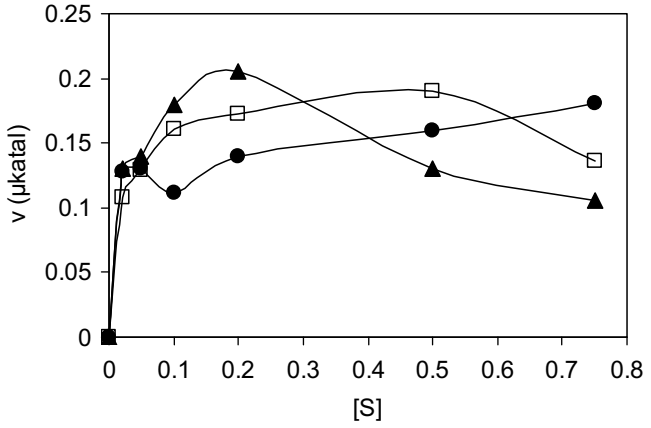

Fig 4. Effect of substrate (PGA) concentration on the activity of different isoforms of PG. (□), (●) and (▲) are PG I, PGII and PGIII, respectively. K_m and V_{max} for PGA for PGI was found to be 0.02% and 0.2 μmoles galacturonic acid formed s^{-1}, respectively.

3.7. Effect of reducing agents

All reducing agents namely βME, DTT and cysteine activated all the isoforms of PG, DTT being the most potent while βME least amongst these. Thus, βME slightly stimulated all PG. PGI and PGIII were highly activated by DTT while PGII only slightly. Cysteine significantly stimulated PGIII but slightly stimulated PGI, PGII (Table 2).

3.8. Effect of substrate concentration on PG isoforms

Effect of substrate (PGA) concentration (0.02–0.75%) on the activity of all the isoforms of PG were investigated. Results are shown in Fig. 4. PGI and PGIII showed inhibition at high substrate concentrations (0.5% and 0.2%, respectively) while PGII did not exhibit inhibition at high substrate concentration. K_m and V_{max} for PGA was calculated using Lineweaver–Burk plot and was found to be 0.02% and 0.2 μmoles galacturonic acid formed s^{-1} for PGI. Since PGII and PGIII did not follow a typical Michaelis–Menten kinetics, K_m and V_{max} for PGII and PGIII was not calculated.

4. Discussion

Dashehari mango PG was found to be expressed as three distinct isoforms during ripening of the fruit. Three isoforms of PG namely PGI, PGII and PGIII were isolated and purified to homogeneity. PGI and PGII were purified 22 folds with specific activities of 4 and 4.15, respectively. PGIII was purified 26 folds with specific activity of 4.75. Three isoforms of PG (PGI, PGII, PGIII) have been reported from another cultivar of mango namely, Alphonso, purified to 19.4, 17, 16.6, folds with specific activities of 34.36, 30, 29.38 for PGI, PGII and PGIII, respectively (Prasanna et al., 2006). Three isoforms of PG have also been reported from fruits like banana and strawberry (Nogata et al., 1993; Pathak & Sanwal, 1998). Fruits like avocado, tomato, peach and pear exhibited only two isoforms (Pressey & Avants, 1973a; Pressey & Avants, 1973b; Pressey & Avants, 1976; Wakabayashi & Huber, 2001). Mango PG isoforms differ with respect to their affinity for ion exchange matrices. Thus, PGI did not bind to DEAE cellulose while PGII and PGIII bind to it.

The three isoforms of Dashehari mango differed in various physicochemical properties amongst themselves as well as from those of Alphonso mango and other fruits. Thus, native molecular weights of Dashehari mango PG isoforms were found to be higher (PGI-120, PGII-105, PGIII-65 kDa) than those of Alphonso mango (PGI-40, PGII-51, PGIII-45 kDa, Prasanna et al., 2006). In banana, PGI, PGII and PGIII were of 23.2, 58, 130 kDa, respectively (Pathak & Sanwal, 1998). Isoforms of PG in other fruits such as avocado (48, 46 kDa, Wakabayashi & Huber, 2001), tomato (84, 44 kDa; 100, 42 kDa; Pressey & Avants, 1973b; Tucker, Robertson, & Grierson, 1980), peach (68, 41 kDa, Pressey & Avants, 1973a) and strawberry (52 kDa, Nogata et al., 1993) were found to be smaller in size as compared to PGI and PGII of Dashehari mango. Furthermore, the subunit structure elucidation of Dashehari mango revealed all the isoforms to be dimeric with PGI being homodimer while PGII and PGIII heterodimers. PG isoforms of Alphonso variety of mango were found to be monomeric ones. PG isoforms reported from other fruits like avocado, tomato and mango were also monomeric (Prasanna et al., 2006; Pressey & Avants, 1973b; Wakabayashi & Huber, 2001).

Dashehari mango PG isoforms also differed in their pH vs. activity profile when compared with those of Alphonso mango PG. Thus, all the three isoforms of Alphonso mango exhibited pH optima at more acidic zone namely, 3.2, 3.6, 3.9 (Prasanna et al., 2006). In comparison to this, Dashehari mango PG isoforms exhibited pH optima 5.0 (PGI) and 6.0 (PGII and PGIII). All the three isoforms exhibited almost negligible activity at pH 3.5. Three isoforms of PG from banana also exhibited lower pH optima (3.3, 3.7, 4.3; Pathak & Sanwal, 1998) than those of Dashehari mango PG isoforms. However, Dashehari mango isoforms exhibited pH profile similar to those of avocado (pH optima of 6.0, Wakabayashi & Huber, 2001), peach, pear and strawberry (pH optima of 5.5, Nogata et al., 1993; Pressey & Avants, 1973a; Pressey & Avants, 1976).

PG isoforms from Dashehari mango also differed with respect to temperature optima. Thus PG I and PGII exhibited temperature optima of 37 °C while PGIII of 40 °C. Alphonso mango showed temperature optima of 37 °C for PGI and PGIII while 42 °C for PGII. Temperature optimum for Zebda variety (Egyptian) mango PG was reported to be between 30 and 35 °C (Labib, El-Ashwah, Omran, & Askar, 1995). Banana PGIII, however, showed temperature optimum 40 °C (Pathak, Mishra, & Sanwal, 2000). The three isoforms of Dashehari mango PG differed with respect to their stability towards heat, PGIII being the most and PGII the least heat stable, respectively. Differences in heat stability amongst multiple forms of PG have also been reported from tomato. Tomato PGI and PGII lost their 50% activity at 78, 57 °C and 100% at 95 and 65 °C, respectively. (Pressey & Avants, 1973b). Zebda variety (Egyptian) mango PG was found to be heat-labile, being unstable at temperature beyond 25 °C (Labib et al., 1995).

Dashehari mango PG activity was slightly inhibited or negligibly affected by the monovalent metal ions. However, banana and tomato PG are reported to be activated by monovalent ions like Na^+, K^+ (Pathak & Sanwal, 1998; Pressey & Avants, 1973b). Peach polygalacturonase also showed inhibition by monovalent ions (Pressey & Avants, 1973a) as observed for Dashehari mango PG. Amongst polyvalent ions, Fe^{+3} was most potent activator of all

the PG isoforms of Dashehari mango. On the contrary, Alphonso mango isoforms were reported to be inhibited by Fe^{+3} (Prasanna et al., 2006). Similar report of inhibition of PG isoforms by Fe^{+3} is also available from banana fruit (Pathak & Sanwal, 1998). Divalent Ca^{+2} also activated PGI and PGII while inhibited PGIII of Dashehari mango. A similar report of activation of PG by Ca^{+2} is available for banana as well as Alphonso mango (Pathak & Sanwal, 1998; Prasanna et al., 2006). One interesting observation was activation of all the isoforms of Dashehari mango by EDTA. The basis of such activation by EDTA is not known, at present, but a report of activations of PG of banana by EDTA is available (Pathak & Sanwal, 1998). With respect to effect of EDTA on PG activity, Dashehari mango PG isoforms were found to be different from those of Alphonso mango where EDTA inhibited all the isoforms of PG (Prasanna et al., 2006).

Amongst reducing agents, DTT was most potent activator of all the isoforms of PG from Dashehari mango followed by cysteine and βME. Enhanced activity of the enzymes in presence of these agents suggest that the reduction of –SH groups improves their performances. However, these results do not allow distinguishing if these groups are involved in the catalytic site, or if they belong to other part of the protein, probably related to disulfide bonding and then influencing its secondary structure. Similar report of activation of PG by reducing agents is available from banana fruit (Pathak et al., 2000).

K_m for PGA for PGI of the Dashehari mango was found to be 0.02%. Low K_m value suggested high affinity of this isoform for its substrate. Alphonso mango PG isoforms also showed K_m for PGA in lower range (0.022–0.025% PGA) similar to Dashehari mango PG (Prasanna et al., 2006). Also Zimbabwean fruit (*Uapaca kirkiana, Zizphus mauritiana, Tamarindus indica* and *Berchemia discolor*), PG showed K_m for PGA in this range (Muchuweti, Moyo, & Mushipe, 2005). However, PG isoforms of banana exhibited higher K_m values for PGA namely 0.22, 0.14 and 0.15% for the three isoforms (Pathak & Sanwal, 1998; Pathak et al., 2000).

The three isoforms of PG from Dashehari mango differed with respect to effect of substrate on enzyme activity. Thus, PGI and PGIII exhibited inhibition at high substrate concentrations, PGIII was more sensitive than PGI to high substrate inhibition. On the other hand PGII was not inhibited by high substrate concentrations. The differential response of Dashehari mango PG isoforms to PGA concentration may be a regulatory advantage during ripening of mango fruit. Thus, in the sequence of enzymes responsible for cell wall loosening, during fruit ripening, first of all pectin methyl esterase (PME) de-esterifies polyuronides (pectin) by removing methyl groups and generates the substrate for next enzyme in the sequence namely, PG. During ripening of tomato fruit, activity of PME have been reported to increase during the early period of ripening and then to decline in the later period when the activity of PG starts increasing (Harriman, Tieman, & Handa, 1991). A similar report of enhanced activity of PME during initial stages of ripening followed by a subsequent increase in PG during later stages of ripening is available from Alphonso variety of mango (Yashoda, Prabha, & Tharanathan, 2007).

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