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Characterisation of an α-galactosidase with potential relevance to ripening related texture changes

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Abstract

α-Galactosidase (EC 3.2.1.22) from ripe papaya (*Carica papaya* L.) fruit was fractionated by a combination of ion exchange and gel filtration chromatography into three forms, viz., α-galactosidase 1, 2 and 3. The predominant isoform, α-gal 2, was probably a tetramer with a native molecular mass of about 170 kDa and 52 kDa-sized subunits and an estimated p*I* of 7.3. The subunit's N-terminal amino acid sequence shared high identity (97%) with the deduced sequence of a papaya cDNA clone (accession no. AY25329) encoding a putative α-galactosidase PAG2 as well as with an *Ajuga reptans* L. *GGT1* clone (accession no. AY386246) encoding a galactan: galactan galactosyltransferase (66%). During ripening, α-galactosidase activity increased concomitantly with firmness loss and this increase was largely ascribed to α-gal 2. The protein level of α-gal 2 as estimated by immunoblot was low in developing fruits and generally increased with ripening. α-Galactosidase 2 also had the ability to markedly catalyse increased pectin solubility and depolymerisation while the polymers were still structurally attached to the cell walls mimicking, in part, the changes that occur during ripening. The close correlation between texture changes, α-gal 2 activity and protein levels as well as capability to modify intact cell walls suggest that the enzyme might contribute to papaya fruit softening during ripening. The purported mechanism of α-gal 2 action as a softening enzyme was discussed in terms of its functional capacity as a glycanase or perhaps, as a transglycosylase.

Keywords: *Carica papaya* L.; Caricaceae; Fruit; α-Galactosidase; Characterisation; Texture; Pectin modification; Cell wall modification

1. Introduction

α-Galactosidase (EC 3.2.1.22), a widely distributed enzyme in the plant kingdom, was reported to occur in actively growing as well as in fully developed leaves and fruits, and also in seeds and tubers (Dey and del Campilo, 1984; Keller and Pharr, 1996). The enzyme exists in multiple forms, having either acidic or alkaline pH optima. Depending on the isoform type, the enzyme may be distributed in the cytosol as well as the vacuole, and in some reported cases also, the cell wall (Keller and Pharr, 1996). The enzyme acts as a hydrolase, releasing free galactose from naturally occurring galactosyl-sucrose oligosaccharides such as raffinose and stachyose, as well as other α-galactosides such as galactolipids and galactoproteins. One noted function of the enzyme is in the mobilisation of α-D-galactosyl residues stored within the raffinose family of oligosaccharides (RFO) and other storage polysaccharides during germination and sprouting of seeds and tubers (Keller and Pharr, 1996).

Other major roles of the enzyme are in phloem unloading and assimilate partitioning in developing fruits, leaves, and tubers of those plant species that translocate RFO (Keller and Pharr, 1996; Gao and Schaffer, 1999). In these sink tissues RFO metabolism occurs primarily via hydrolysis by α-galactosidase that acts in tandem with acid

Abbreviations: AIS, alcohol-insoluble solid; GGT, galactan: galactan galactosyltransferase; PNPG, *p*-nitrophenyl-α-D-galactopyranoside; RFO, Raffinose family of oligosaccharides; UA, uronic acid.

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invertase and other hydrolases (Madore, 1995; Keller and Pharr, 1996). More recently, galactan: galactan galactosyltransferase, an enzyme that is involved in the biosynthesis of the long-chain RFO and which seems to posses certain molecular and biochemical characteristics as a α-galactosidase, had been reported (Haab and Keller, 2002). This vacoular enzyme plays a role in RFO accumulation and storage (Bachmann et al., 1994).

The presence of α-galactosidase in ripening fruits of plants that are not known to translocate RFO was also reported, however, the significance of this observation to ripening related texture changes is unknown (Ali et al., 1995; Gross et al., 1995; Trinchero et al., 1999). In olive, a correlation between α-galactosidase activity and changes in the sugar composition of the cell wall during ripening was established (Heredia et al., 1993; Fernandez-Bolanos et al., 1995). Likewise, in ripening papaya, besides β-galactosidase, α-galactosidase activity also correlated closely with firmness loss (Ali et al., 1998). The roles played by β-galactosidases in fruit softening based on their functional capability as exo-β-galactanases have been suggested (Rose and Bennett, 1999; Smith et al., 2002; Ali et al., 2004; Lazan et al., 2004). Unlike β-galactosidase, the significance of α-galactosidase as a cell wall modifying enzyme is still uncertain, inasmuch as efforts to characterise the enzyme have hitherto been limited to few fruits (Pressey, 1984; Itoh et al., 1986; Burns, 1990; De Veau et al., 1993). However, there are reports suggesting that α-galactosidase isolated from various plant and microbial sources have transglycosylation activities (Dey, 1979; Kato et al., 1982; Mitsutomi and Ohtakara, 1988; Hashimoto et al., 1995; Koizumi et al., 1995; Van Laere et al., 1999) – a catalytic property that might be relevant to cell wall modification during fruit growth and development. The aim of this study was to isolate and characterise α-galactosidase 2, a predominant α-galactosidase isoform from ripe papaya, and to assess its possible contribution to fruit softening during ripening. The enzyme was found to have significant β-galactanase as well as pectin hydrolysing activities but devoid the activity as a β-galactosidase.

2. Results and discussion

2.1. Purification and characterisation of papaya α-galactosidase

A single, high-salt extract of ripe papaya was precipitated at 0–85% ammonium sulphate saturation and dialysed against 0.1 M sodium acetate buffer at pH 5.2 before being subjected to a CM-Sepharose CL-6B, which acts as the first chromatographic step. The ammonium sulphate precipitation not only served to concentrate the enzymes at initial stage of purification, but also gave rise to a purification factor of 3.15 (Table 1). This step managed to get rid of about 66.7% total protein while recovering all (104.8%) the enzyme activity. The subsequent cation exchange chromatography can resolve α-galactosidase to three forms, designated as α-gal 1, 2 and 3 in accordance with their sequential elution from the column (Fig. 1). α-Galactosidase 1 was eluted in the flow-through, while α-gal 2 and 3 were step-eluted with the addition of 0.05 and 0.3 M NaCl, respectively, to the eluting buffer, and were purified to 0.5-, 31.6- and 7.8-fold (Table 1). The low purification factor for α-gal 1 was most probably contributed by contaminating proteins that co-eluted with the enzyme. α-Galactosidase 1 then reintroduced to the cation exchange column at a lower pH (pH 4.0) and the bound enzyme eluted at 0.1 M NaCl (Fig. 2(a)). This step raised the purification factor of this isoform to 43.6 (Table 1). α-Galactosidase 2, however, need to be resolved through

Table 1
Purification table of α-galactosidase from ripe papaya fruit tissues

Purification stage	Total volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat mg^{-1})	Recovery percentage (%)	Purification factor
Crude extract	704	1387	251	5.5	100	1.0
Ammonium sulphate (0–85%) precipitation	86	1454	83.4	17.4	104.8	3.15
CM-Sepharose (pH 5.2)						
α-Galactosidase 1	173	92.8	32.4	2.9	6.7	0.52
α-Galactosidase 2	164	1284	7.4	175	92.6	31.6
α-Galactosidase 3	126	233	5.4	43.3	16.8	7.8
Ammonium sulphate (0–85%) precipitation of α-galactosidase 1	31.3	65.7	14.7	4.5	4.74	0.81
CM-Sepharose (pH 4.0)						
α-Galactosidase 1	73.7	52.1	0.22	241	3.76	43.6
Sephacryl S-200						
α-Galactosidase 2	31.0	425.4	1.5	280.1	30.7	50.6
Sephadex G-75						
α-Galactosidase 2	13.2	197.7	0.23	887.6	14.3	160.5
α-Galactosidase 3	36.0	48.4	1.38	35.0	3.5	6.3

Experiments repeated at least three times and typical results shown.

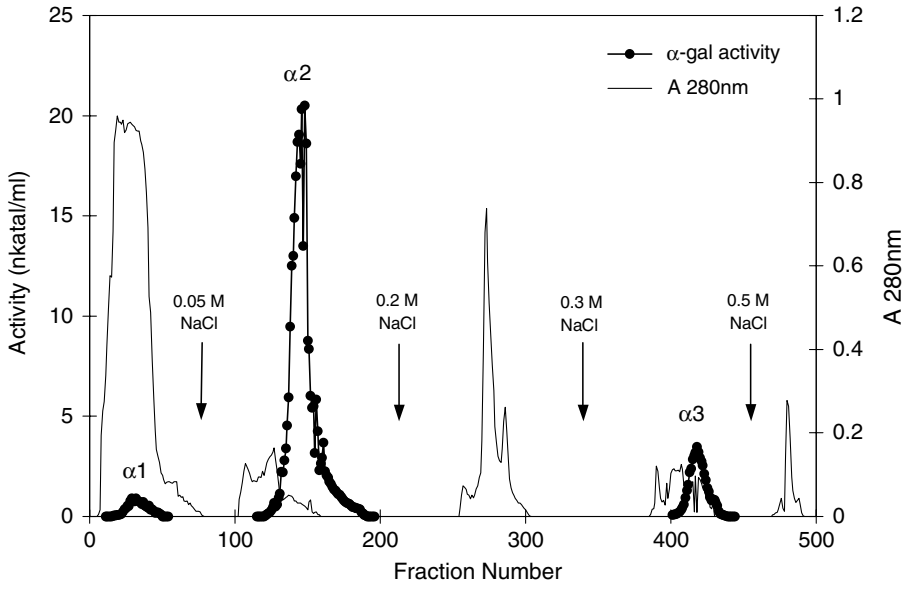

Fig. 1. Elution profile of α-galactosidase activity and absorbance at 280 nm from CM-Sepharose CL-6B column at pH 5.2. α1, α2 and α3 represent α-gal 1, 2 and 3, respectively.

two gel filtration steps, viz. Sephacryl S-200 (Fig. 2(b)) and Sephadex G-75 (Fig. 2(c)) columns, to remove residual protein contaminants. Fractions that contained considerably high activity of α-gal 2 was sacrificed in order to remove contaminating proteins, notably the β-galactosidases, and resulted in relatively low recovery. Nevertheless, α-gal 2 was successfully purified 160-fold via the purification scheme (Table 1). Meanwhile, α-gal 3 was loaded onto a Sephadex G-75 column (Fig. 2(d)). A marked reduction in the activity of α-gal 3 during the purification procedure suggested that the enzyme in the preparation was relatively unstable (Table 1).

The native molecular masses of α-gal 1, 2 and 3 as estimated by gel filtration chromatography on a Sephadex G-150, was about 57, 170 and 29 kDa, respectively. Native PAGE analyses of the α-galactosidase isoforms showed single protein band for both α-gal 2 and 3, respectively (Fig. 3(a)). Both α-gal 2 and 3 also appeared as single protein band upon SDS–PAGE with estimated sizes at 52 and 29-kDa (Fig. 3(b)) and upon isoelectric focusing, their respective p*I* values were estimated at 7.2 and 9.3 (Fig. 3(c)). α-Galactosidase 1 could not be visualised on the gels due to low protein content as the fraction were too diluted even after the use of micro-concentrator while α-gal 3, the isoform was very unstable in relatively pure form. α-Galactosidase 2 appeared to be a polymeric, approximately 170-kDa protein comprising 52-kDa subunits. This papaya enzyme was probably a glycoprotein, as it stained positively with PAS in both native and SDS–PAGE (data not shown). As SDS–PAGE analysis might overestimate the molecular size of glycoproteins (Segrest and Jackson, 1972), it seemed probable that the native protein of α-gal 2 was a tetramer. Tetrameric α-galactosidases were also found in albeit, a limited numbers of plant species, which include, among others, coffee, soybean, broad beans and mung bean. Notable properties of tetrameric α-galactosidases were their lectin-like and phytohemagglutinin activities (Dey and del Campilo, 1984; Dey and Pridham, 1986).

Fig. 3(d) shows the N-terminal amino acid sequence of the 52-kDa α-gal 2 subunit. BLAST analysis of the first 30 amino acid residues of the α-gal 2 subunit gave the highest score with deduced amino acid sequence of the putative protein encoded by a papaya α-galactosidase *PAG2* (accession no. AY253299), sharing about 97% identity, and followed by α-galactosidases encoded by cDNA clones from coffee bean (*Coffea arabica*), 80%, tomato (*Lycopersicon esculentum*), 72%, soybean (*Glycine max*), 70%, kidney bean (*Phaseolus vulgaris*), 66%, and guar (*Cyamopsis tetragonoloba*), 64%. All these matches belong to the glycosyl hydrolase family 27 (Henrissat and Romeu, 1995). However, the N-terminal sequence of the α-gal 2 subunit also shared significant identity (66%) with the deduced sequence of a putative galactan: galactan galactosyltransferase (encoded by *GGT-1*; accession no. AY386246), the enzyme involves in the biosynthesis of long-chain RFO in *Ajuga reptans* L. (Fig. 3(e)).

The kinetic of α-gal 1 activity was found to be Michaelian with K_m and V_{max} values of 1.36 mM and 1.66 U mg^{-1} protein, respectively. α-Galactosidase 2 and 3, however, exhibited substrate inhibition phenomenon when α-PNPG substrate concentration exceeded 4 mM. This deviation from simple kinetics experienced by the two isoforms was dissimilar from uncompetitive inhibition; the enzymes' activity instead of becoming zero at high substrate concentrations, becomes constant at a value below the maximal activity (Fig. 4). Similar observations made with α-galactosidases from a number of plant and other

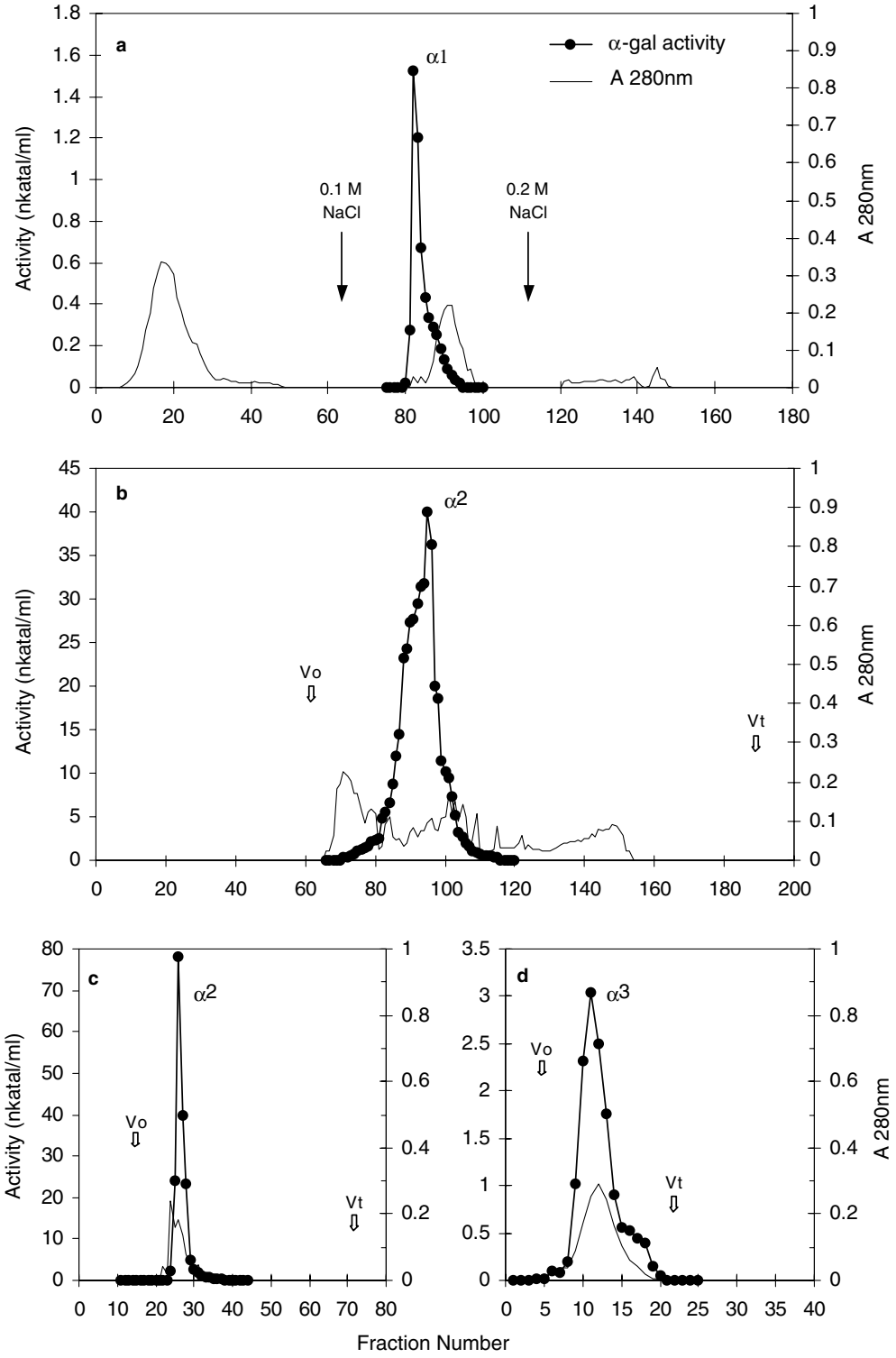

Fig. 2. Elution profile of α-gal 1 (fraction 24–40 of CM-Sepharose CL-6B column, pH 5.2) from CM-Sepharose CL-6B column at pH 4.0 (a), α-gal 2 (fraction 138–186 of CM-Sepharose CL-6B column, pH 5.2) from Sephacryl S-200 column (b), α-gal 2 (fraction 72–96 of Sephacryl S-200 column in b) from Sephadex G-75 column (c) and α-gal 3 (fraction 421–441 of CM-Sepharose CL-6B column, pH 5.2) from Sephadex G-75 column (d). V_o and V_t represent the void volume and the total column volume, respectively.

sources (Dey and del Campilo, 1984). Apparent K_m values of α-gal 2 and 3 were at 1.35 and 1.09 mM, while their apparent V_{max} values were, respectively, at 4.2 and 3.9 U mg^{-1} protein. α-Galactosidase 1, 2 and 3 had optimal assay pH of 5.5, 3.0 and 3.5, and assay temperature of 65, 45 and 35 °C, respectively. Heat stability studies showed that the respective α-galactosidase isoforms lost 50% activity when exposed for 10 min at 55, 47 and 48 °C.

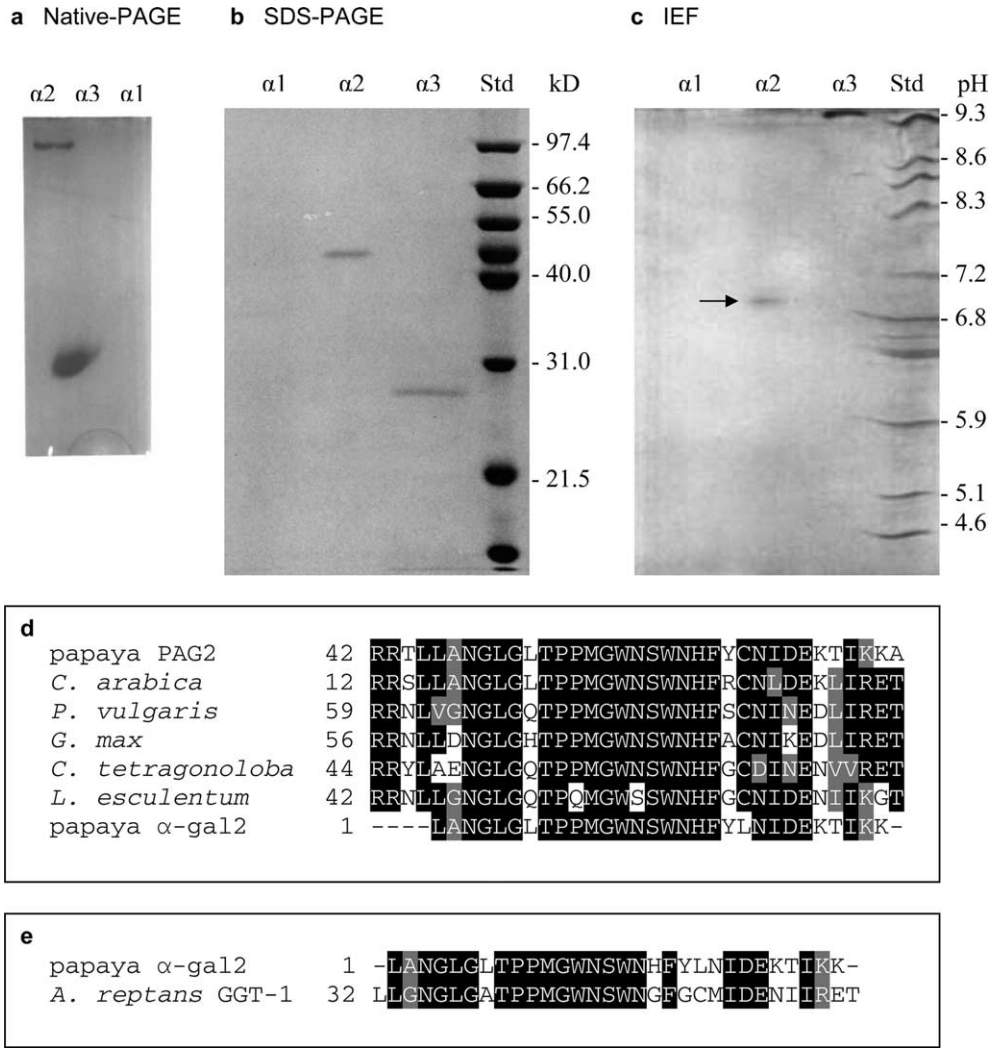

Fig. 3. Native-PAGE (a), SDS–PAGE (b), and Ampholine® PAGplate (pH 3.5–9.5) isoelectric focusing electrophoresis of α-galactosidase isoforms (c). Arrow indicates the position of α-gal 2 while standard protein markers for the SDS–PAGE and isoelectric focusing gel are shown in b and c. α1, α2 and α3 represent α-gal 1, 2 and 3, respectively. Alignment of N-terminal amino acid sequence of α-gal 2 with α-galactosidase from *C. papaya* (GenBank accession no. AY253299), *C. arabica* (GenBank accession no. L27992), *P. vulgaris* (GenBank accession no. U12927), *G. max* (GenBank accession no. U12926), *C. tetragonoloba* (GenBank accession no. X14619) and *L. esculentum* (GenBank accession no. AF191823) (d). Alignment of amino acid sequence of α-gal 2 with GGT-1 from *A. reptans* (GenBank accession no. AY386246) (e). Identical and homologous amino acids are boxed in black and grey, respectively, in d and e.

The effect of cations on the papaya α-galactosidases was also studied (Table 2). The presence of Ca^{2+} at 5–10 mM activated both α-gal 2 and 3 by about 20–30%, while Mg^{2+} seemed to have minimal effects (data not shown). On the contrary, α-gal 2 and 3 were inhibited when K^+ or Na^+ were present at high concentrations (2 M); the enzymes only retained about 21–59% activity. α-Galactosidase 1, on the whole, was not as much affected by K^+ though there was some 9–22% inhibition exhibited when Mg^{2+} (10 mM), Ca^{2+} (10 mM) or Na^+ (2 M) were present. Both Hg^{2+} and Ag^{2+} cations were potent inhibitors of the enzymes' activity, exhibiting total inhibition at 1 mM Ag^{2+} and 2 mM Hg^{2+}, respectively. EDTA seemed to have no inhibitory effect on the isoforms activity (data not shown).

The papaya α-galactosidases also displayed varied sensitivity towards the presence of various neutral sugars (Table 2). They were particularly inhibited by galactose at low concentrations (5–10 mM), retaining about 17–33% activity at the highest concentration tested (Table 2). Such result is typical for α-galactosidases isolated from diverse plant sources (Dey and del Campilo, 1984; Keller and Pharr, 1996). Structural analogues of galactose exhibited different effects on the enzymes. Xylose seemed also an effective inhibitor, causing about 21–36% suppression of the enzymes' activity at 10 mM. Fucose was a moderate inhibitor; it managed to inflict discernible (47–11%) inhibition at a concentration (75 mM) that was about 20 times as high as that of the PNPG substrate (4 mM). Glucose and mannose seemed to have no effect on the enzymes' activity, as was the case with rhamnose and arabinose (data not shown).

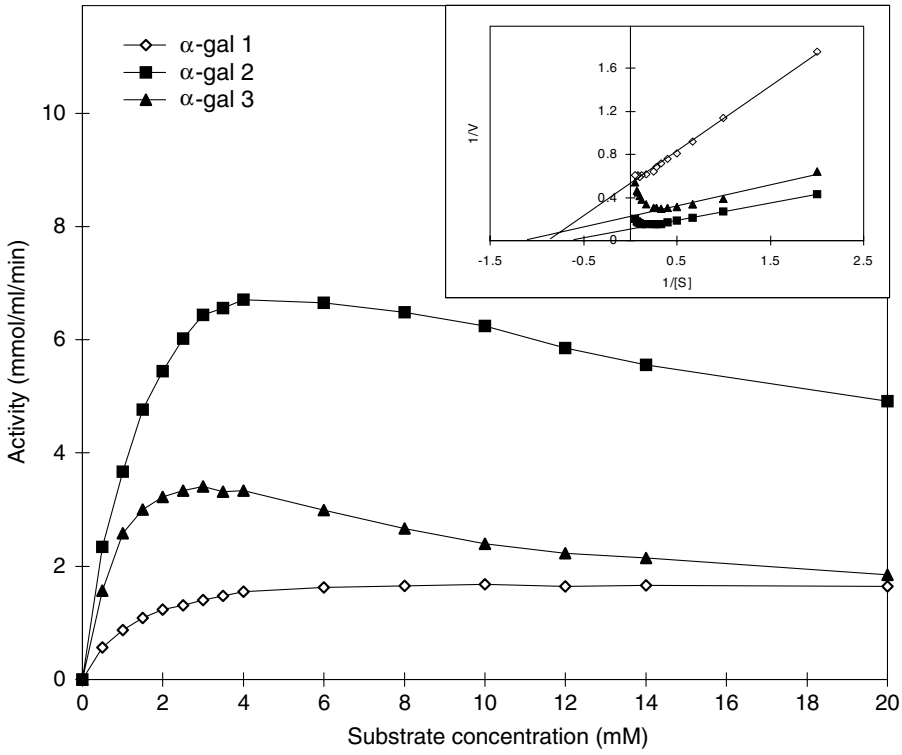

Fig. 4. Effects of PNPG substrate concentration on the activity of α-galactosidase isoforms. Inset is the Lineweaver–Burk plot of the reaction kinetics.

Table 2
Effect of selected cations and sugars on papaya α-galactosidase activity

Cation or sugar	Concentration	Relative activity (%)		
		α-Galactosidase 1	α-Galactosidase 2	α-Galactosidase 3
Control	0	100	100	100
NaCl	0.5 M	97.7	92.7	85.0
	1.0	85.3	59.8	49.1
	2.0	78.1	53.3	21.1
KCl	0.5 M	98.1	99.7	81.8
	1.0	91.8	68.7	53.3
	2.0	91.2	58.7	25.4
$AgNO_3$	0.5 mM	19.2	9.1	14.7
	1.0	0.0	0.0	0.0
$HgCl_2$	0.5 mM	0.0	10.5	30.8
	1.0	0.0	7.9	17.6
	2.0	0.0	0.0	0.0
Galactose	1 mM	65.8	95.2	83.8
	5	30.1	27.0	51.3
	10	18.8	16.6	33.4
Xylose	1 mM	97.0	112.8	105.6
	5	85.6	91.3	92.9
	10	63.8	73.3	79.3
	50	27.1	44.0	68.3
	75	19.5	37.3	55.0
Fucose	1 mM	90.1	107.2	111.5
	50	60.8	81.7	97.1
	75	52.9	74.5	88.9

Experiments repeated three times and a typical result shown.

2.2. Substrate specificity and in vitro cell wall pectin modification

Specificity of the α-galactosidases against several synthetic as well as endogenous galactan and arabinogalactan substrates derived from other plant sources is shown in Table 3. Values as presented in Table 3 are typical results for at least three experiments. All α-galactosidase preparations were free from activities of other glycosidases such as α-arabinosidase, α-mannosidase, β-glucosidase and most notably, β-galactosidase. The present purification protocol seemed efficient in fractionating the isoforms of α- and β-galactosidase such that the latter enzymes, too, were devoid of α-galactosidase. Polygalacturonase (PG) and pectin methylesterase (PME) activities were also undetectable in any of the fractions (data not shown). However, despite devoid the activity as a β-galactosidase, the papaya enzymes, and in particular the major isoform α-gal 2, demonstrated a markedly high ability in hydrolysing galactans, especially that from (1 → 3)β- and (1 → 6)β-linked gum arabic as compared to (1 → 4)β-linked spruce galactans (Table 3). Likewise, the β-galactosidases of ripening papaya have β-galactanase activities (Ali et al., 1998).

As α-gal 2 had the capability in hydrolysing cell wall derived β-galactans, its potentials in modifying structurally intact wall polysaccharides was assessed by incubating (72 h, 37 °C) the purified enzyme with cell wall preparations (AIS) of unripe fruits. Indeed, the enzyme was able to catalyse both increased solubility as well as depolymerisation of the still structurally attached pectin networks (Table 4; Fig. 5(a)). The rate of pectin solubilisation over the three-day incubation period (ca. 0.35 mg UA h^{-1} g^{-1} AIS) was comparable with that occurring in vivo (ca. 0.45 mg UA h^{-1} g^{-1} AIS) during ripening (Table 4). The enzyme was also able to affect increased pectin solubility from the cell walls of unripe carambola fruit, but at a much slower rate (0.06 mg UA h^{-1} g^{-1} AIS) than the rate observed for the papaya fruit (Table 4). EDTA, which does not have any inhibitory effects on the enzyme, increased the pectin level solubilised by α-gal 2 but not its depolymerisation if the chelator was added into the reaction mixture (Table 4; Fig. 5(a) and (b)). Addition of a chelator would disrupt the calcium bridges, and hence, would probably increase the accessibility for enzymes to potential substrates. Instead, a more dramatic polymer size downshift was observed when the chelator was excluded – a treatment which would likely result in retention of structural conformations of the cell wall. It seems, existence of certain polymeric conformation in the native wall is essential for in situ substrate recognition by the enzyme and this would probably explain the inability of the purified enzyme to affect modification of the carambola fruit cell wall as marked as that of papaya's (Table 4). Ability of α-gal 2 to markedly catalysed increased pectin solubility and degradation – changes that are characteristics for ripening papaya (Lazan et al., 2004) – suggests that the enzyme might contribute to texture related changes during ripening of the fruit. The potential of α-galactosidase isolated from citrus and avocado fruits to hydrolyse cell wall materials has been reported (Burns, 1990; De Veau et al., 1993).

Table 3
Substrate specificity of purified α-galactosidase isoforms

Substrate	Enzyme activity (nkat/mg protein)		
	α-Gal 1	α-Gal 2	α-Gal 3
ρ-Nitrophenyl α-D-galactopyranoside	278.3	2408.6	13.8
ρ-Nitrophenyl β-D-galactopyranoside	ND	ND	ND
ρ-Nitrophenyl α-L-arabinopyranoside	ND	ND	ND
ρ-Nitrophenyl β-D-glucopyranoside	ND	ND	ND
ρ-Nitrophenyl α-D-mannopyranoside	ND	ND	ND
Galactan (gum arabic)	910.9	1195.7	158.1
Galactan (spruce)	442.9	896.8	7.8
Arabinogalactan (larchwood)	134.2	210.2	9.4

Experiments repeated three times and a typical result shown.
ND, not detectable.

Table 4
Changes in pectin solubility following incubation of unripe fruit's AIS with purified α-galactosidase 2 for 72 h and in ripening papaya and carambola fruits tissues

Treatment		Soluble polyuronide level (mg uronic acid/g AIS)
A. Papaya		
Without EDTA	Inactive α-Gal 2	160.7 ± 4.2
	Active α-Gal 2	186.0 ± 4.1
	Unripe (day 0)	161.1 ± 4.1
	Ripe (day 8)	247.8 ± 3.1
With EDTA	Inactive α-Gal 2	209.9 ± 4.0
	Active α-Gal 2	248.3 ± 4.1
	Unripe (day 0)	211.1 ± 4.1
	Ripe (day 8)	314.8 ± 4.8
B. Carambola		
Without EDTA	Inactive α-Gal 2	51.1 ± 2.5
	Active α-Gal 2	55.4 ± 2.6
	Unripe (day 0)	51.6 ± 2.3
	Ripe (day 24)	73.5 ± 2.9

Results are means of three fruits or replicates ± SE.

2.3. Potential of α-galactosidase 2 as a ripening related softening enzyme

Changes in α-galactosidase activity and that of its isoforms, resolved by chromatography on a CM-Sepharose CL-6B cation exchange column (Fig. 1) in developing and/or ripening papaya fruits, are shown in Fig. 6. The results indicated that significant α-galactosidase activity was detectable in immature fruits; the enzyme's activity increased as the fruit develops and ripens, registering a dramatic increase at the 25–50%-YR transition stage, which coincided with the period of rapid firmness loss during ripening (Fig. 6(a)). α-Galactosidase 2 was the predominant isoform present even in the unripe, mature and firm 5%-YR fruits, and the enzyme activity increased markedly through ripening, matching closely the increase in the activity of total α-galactosidase. The activity of the other

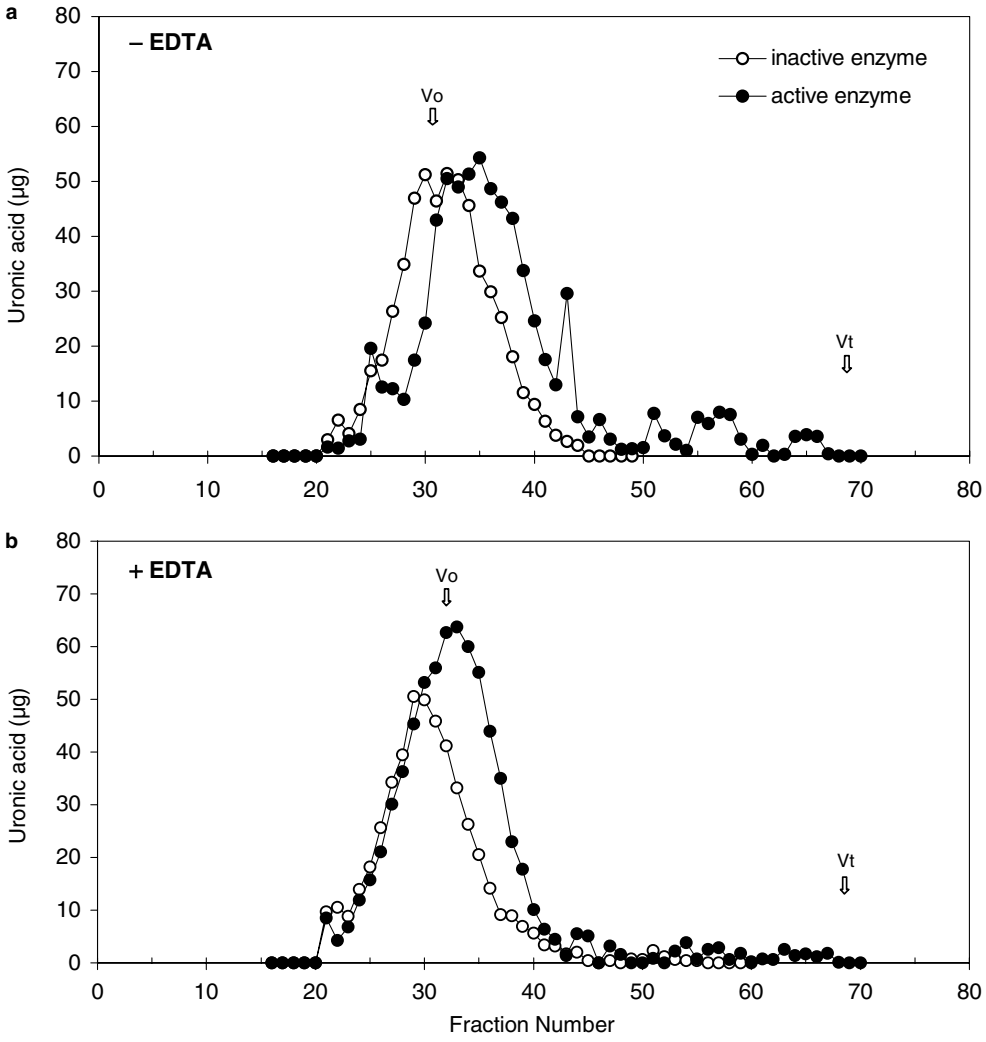

Fig. 5. Sephacryl S-500 elution profile of papaya cell wall polyuronides after a 72 h treatment with α-gal 2 in the absence (a) and presence (b) of EDTA. V_o and V_t represent the void volume and the total column volume, respectively.

two isoforms was very low initially and changed very little as the fruit ripened (Fig. 6(a)).

Changes in the α-gal 2 protein level in developing and ripening papaya fruit were estimated by western blotting analysis of the extractable proteins using IgG1 from polyclonal antibodies raised against the purified enzyme (Fig. 6(b)). Upon quantification of the band's intensity, results as presented in Fig. 6(c) showed that α-gal 2 protein was detectable throughout the maturation stages and increased as the fruit ripened, with a discernibly greater accumulation occurred during the 25–75%-YR transition period. Likewise, the level of extractable proteins increased throughout fruit development (Fig. 6(c)). It appears the increase in the α-gal 2 activity was attributed, at least in part, to its de novo protein synthesis. The dramatic increase in the enzyme's activity, which coincided with the time of rapid firmness loss, coupled with its ability to modify intact cell walls suggest that α-gal 2 might be an important ripening related softening enzyme. In addition, α-gal 2 might also contribute to cell wall modifications during the stages of active growth since its presence was discernible throughout the development of the fruit (Fig. 6(b)).

The mechanism(s) by which α-gal 2 affects modification of the structurally intact cell walls appeared to be related to the enzyme's functional capacity as a glycanase, as was probably the case with the β-galactosidases (Ali et al., 1998; Lazan et al., 2004; Balasubramaniam et al., 2005). The observation that α-gal 2 that devoid the activity as a β-galactosidase was able to hydrolyse β-galactans, were perplexing. Such result could probably mean that the enzyme may have multiple substrates – a catalytic property that might be relevant to polymeric enzymes such as α-gal 2 that comprises several subunits and hence possibly, having multiple active sites. The β-galactosidase from apple fruit shown to have more than one catalytic site for different substrates acting synergistically which was probably in conformity with the functional role of a wall enzyme to modify complex structures as the primary walls (Dick et al., 1990). The isoforms of α- and β-galactosidase seemed

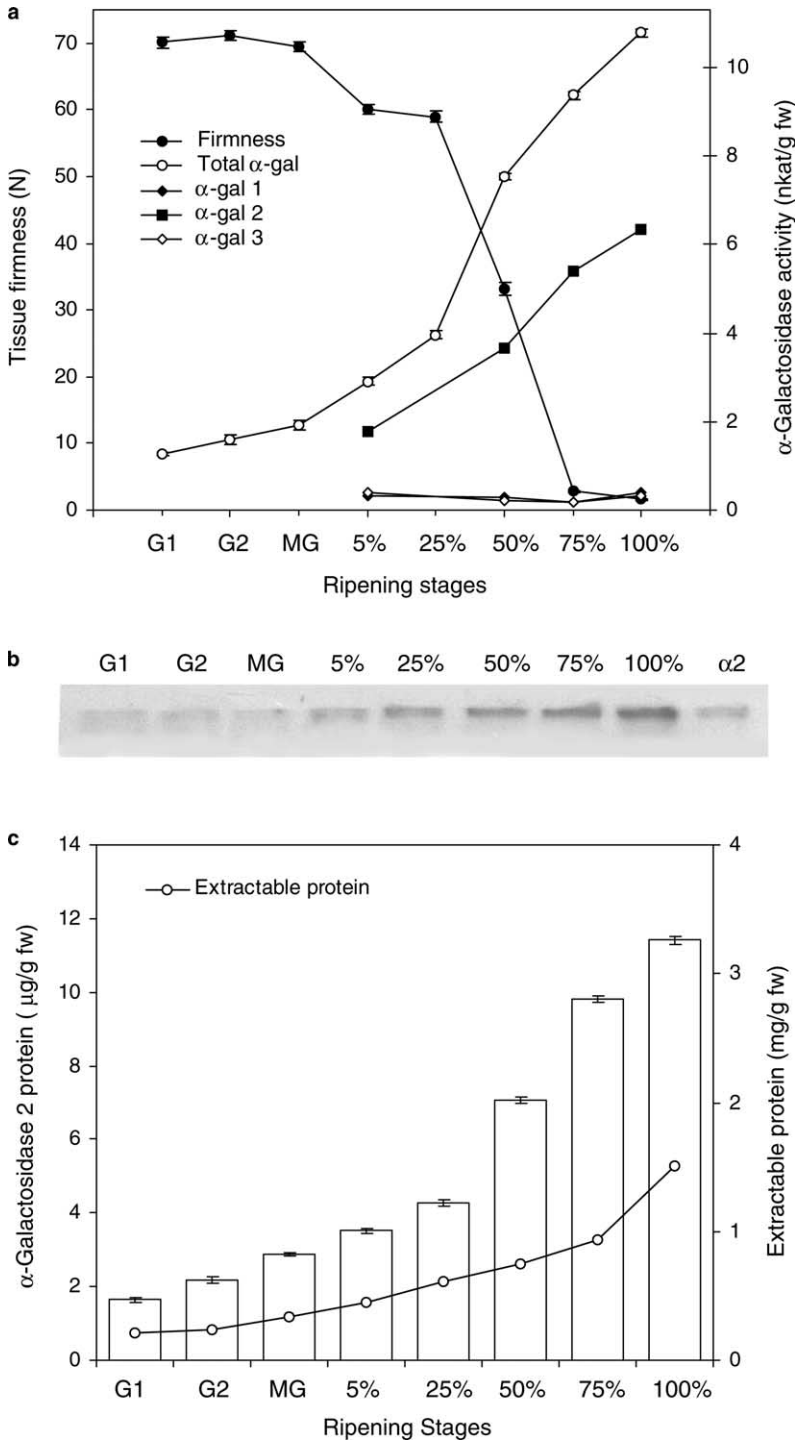

Fig. 6. Changes in tissue firmness (●) and total activity (bigcirc) of α-galactosidase as well as activities of its isoforms α-gal 1, 2, and 3 (◆, ■, ◇) during maturation and ripening of papaya fruit (a). Total protein (about 100 μg), extracted from different stages of developing papaya fruit, were resolved in native-PAGE, blotted onto nitrocellulose membrane and immunodetected with IgG1 of the α-gal 2 at the dilution of 1:500 (b). Levels of extractable protein (○) and α-gal 2 protein (histogram) as estimated from b using a densitometer (c). Results are means ± SE of eight fruits for tissue firmness, total activity of α-galactosidase and extractable protein, and ±SE of four fruits for α-gal 2 proteins, while that of the α-galactosidase isoforms activity are an average of two experiments.

to represent distinct class of wall enzymes, as the molecular evidence of clones encoding papaya PAG2 (accession no. AY253299) and PBGII (accession no. AF064786) suggest (Othman et al., 1998; Jamal, 2004). Catalytically, as a β-glycanase, the former enzyme appeared more potent than the latter (Table 3; Ali et al., 1998). However, it is important to carry out studies using tailor-made galactan oligosaccharides and enzyme end-products analysis to really understand the reaction mechanism of papaya α-gal 2. In the in vitro modification study (Table 4; Fig. 5), the peak

activity fractions (Fig. 2(c)) as used were free of β-galactosidase (Table 3), thereby, ruled out the possibility that contaminating β-galactosidases might have caused the observed changes. If indeed there were undetectable residual activities, their levels would be much too low to have any discernible impact on the intact wall.

The endogenous substrates that would likely be the target of α-gal 2 were galactans and/or arabinogalactans. Our in vitro cell wall modification studies involving sequential extraction of cell wall fractions in water, CDTA, sodium carbonate, and in dilute and strong alkali provide evidence that papaya α-gal 2 has the ability to simultaneously modify not only tenaciously bound pectin, but also xyloglucan-containing hemicelluloses (data not shown). The purified enzyme was also able to transfer part of the wall xyloglucan from the hemicellulosic to the pectin fractions. All these observations suggest the possible existence of tight structural linkages in the papaya fruit's cell wall between the pectin and the xyloglucan–cellulose networks as envisaged by the Albersheim wall architectural model, with galactans and/or arabinogalactans probably serve as the glycan cross-links (Keegstra et al., 1973; Lazan et al., 2004). Ability of α-gal 2 to degrade synthetic PNPG substrate suggests that it might act as an exo-glycosidase. However, its ability to significantly modifying the intact wall could not discount the possibility that the enzyme might also function as an endo-glycanase.

With respect to the biochemical mechanism of α-gal 2 as a softening enzyme, biochemical as well as molecular evidence seems to suggest another possibility. There were reports that glycosidases such α- and β-galactosidase isolated from numerous prokaryotic and eukaryotic sources have transglycosylation activities (Dey, 1979; Kato et al., 1982; Dey and del Campilo, 1984; Mitsutomi and Ohtakara, 1988; Hashimoto et al., 1995; Koizumi et al., 1995; Yoon and Ajisaka, 1996; Van Laere et al., 1999). The *PAG2* clone encoding a putative papaya α-galactosidase whose deduced amino acid sequence almost matched perfectly (97% identity) the N-terminal sequence of the purified α-gal 2 subunit (Fig. 3(d)), contains conserved domains (70% identity) that were characteristics for mature polypeptide of a galactan: galactan galactosyltransferase encoded by *GGT1* (Jamal, 2004). The N-terminal sequence of the purified enzyme subunit, too, showed homologies (66% identity) with the deduced sequence of the putative *Ajuga reptans* GGT1 (Fig. 3(e)). Thus, a possibility is raised that the wall modifying ability of α-gal 2 might probably be attributed to its activity as a galactan transglycosylase/hydrolase, perhaps in a manner comparable with the catalytic function that of the wall enzyme, xyloglucan endotransglucosylase/hydrolase (Rose et al., 2002). Ability of α-galactosidases to affect modification of architecturally complex cell walls as a transglycosylase rather than simply as a hydrolase deserve further investigation because it brings fresh insight into the manner how cell walls are disassembled during ripening, at least in papaya.

3. Experimental

3.1. Plant material and sampling

Carica papaya L. cv. Eksotika fruits purchased from a private farm in the Selangor state of West Malaysia at the 5% yellow ripe (YR) stage were stored at ambient (28 °C) temperature to ripen. Whenever needed the fruits were sampled when they attained the 25%, 50%, 75% and 100%-YR stages. In certain experiments, fruits at maturation stages G1 (immature green 1), G2 (immature green 2) and MG (mature green) as defined in Ali et al. (1998), were also purchased from the same farm. Procedures for fruits handling and sampling as well as texture determination were as described in Lazan et al. (1995). Texture was determined ($n = 8$) before the tissue was frozen in liquid nitrogen and kept at -70 ° C.

3.2. Enzyme extraction

Extraction procedure was as described by Ali et al. (1998). All steps were done at 4 °C. Frozen mesocarp tissues (50 g) from each developmental stage ($n = 8$) were retreated with liquid nitrogen, ground to fine powder and subsequently homogenised in (50 ml) citrate buffer (0.1 M) pH 4.6 containing NaCl (1 M), disodium EDTA (13 mM), PVP (1%) and β-mercaptoethanol (10 mM), using dry and wet mills, respectively, on a National MX-791 S blender. Homogenate was left for 1 h with occasional stirring followed by centrifugation at 13,000*g* (SORVALL SUPERSPEED 5B) for 30 min. The supernatant recovered for enzyme assay, protein estimation and Western blotting analysis.

3.3. Enzyme activities and protein determination

α-Galactosidase was assayed in a reaction mixture that contained 0.52 ml citrate buffer (0.1 M) at pH 5.5 (for α-gal 1) or pH 3.0 (for α-gal 2 and 3), 0.40 ml bovine serum albumin (0.1%) (Sigma) and 0.40 ml *p*-nitrophenyl-α-D-galactopyranoside (PNPG) (Sigma) at 6 mM (for α-gal 1) or 4 mM (for α-gal 2 and 3) incubated at 37 °C for 10 min before 0.08 ml enzyme was added. After 15 min of incubation, the reaction stopped by addition of 2.0 ml sodium carbonate (0.2 M) and absorbance at 415 nm was measured. Sample boiled for 10 min used as control. Similar procedures, as described by Ali et al. (1998), were used to assay for α-galactosidase in crude and semi-pure sample, and β-D-galactosidase, α-D-mannosidase, β-D-glucosidase and α-L-arabinosidase in the purified α-gal 1, 2 and 3 fractions. Polygalacturonase (PG) and pectin methylesterase (PME) activity was estimated by the method described in Lazan et al. (1995). All enzyme activities except PME, expressed as nkatal g^{-1} fresh weight, while PME expressed as nequivalent g^{-1} fresh weight s^{-1}. Protein concentration was determined either by the Bradford (1976) method with bovine serum albumin as a standard, or by absorbance at

280 nm. Protein in crude extracts was precipitated with equal volume of 10% (w/v) trichloro acetic acid (TCA), rinsed twice with 5% TCA and dissolved in 0.1 M NaOH prior to protein determination (Bradford, 1976).

3.4. α-Galactosidase purification

All steps were conducted at 4 °C. Crude extract was prepared from 500 g 100%-YR stage papaya fruit tissue and the protein was precipitated by addition of ammonium sulphate at 0–85% saturation. The mixture was left for 30 min followed by centrifugation (13,000*g* for 30 min). Protein pellet was dissolved in minimal 0.1 M sodium acetate buffer (pH 5.2) and dialysed using a 12 kD molecular weight cut-off dialysis tube (Sigma), against the same buffer containing 1 mM dithiotreitol (DTT) for 20–22 h. The sample was then applied to a CM-Sepharose CL-6B (Pharmacia) column (30 cm × 2.5 cm) previously equilibrated with the above buffer. Elution was done at 20 ml h^{-1} with the same buffer until absorbance at 280 nm reached zero, followed by sequential elution with increasing NaCl concentration (0.05, 0.2, 0.3 and 0.5 M) in the elution buffer. Fractions (6.8 ml) assayed for α-galactosidase and three activity peaks noted. Each peak was pooled, precipitated in ammonium sulphate (0–85%) and dissolved individually in a minimum volume of corresponding buffer to be used in the subsequent step.

The first peak of α-galactosidase activity (α-gal 1) was dialysed against 0.1 M acetate buffer (pH 4.0), containing DTT (1 mM) for 20–22 h and applied to a CM-Sepharose CL-6B (30 cm × 2.5 cm) column pre-equilibrated with the same buffer at the rate of 20 ml h^{-1} and elution was carried out until 280 nm absorbance reached zero. Sequential elution was performed with 0.1 and 0.2 M NaCl in the same buffer and the α-galactosidase activity (α-gal 1) was eluted in the 0.1 M NaCl. Meanwhile, the second peak of α-galactosidase (α-gal 2) was loaded onto a Sephacryl S-200 (Pharmacia) column (52 cm × 2.5 cm), previously equilibrated with 0.1 M acetate buffer (pH 5.2) and eluted at the rate of 40 ml h^{-1}. Fractions containing the enzyme's activity pooled, concentrated (0–85% ammonium sulphate saturation) and applied to a Sephadex G-75 (Pharmacia) column (22 cm × 2.5 cm), equilibrated with the same buffer. The enzyme eluted as a single peak from this column. The third activity peak (α-gal 3) from CM-Sepharose CL-6B column was also loaded onto the same Sephadex G-75 column equilibrated with the same buffer as above and eluted as a single peak. The highest activity peak fractions for each isoform used in enzyme characterisation studies.

3.5. Enzyme characterisation

Molecular masses of enzymes determined by elution from a Sephadex G-150 (Pharmacia) column (68 cm × 1.5 cm) with 0.05 M potassium phosphate (pH 7.0) buffer, which contained 0.15 M NaCl and 0.02% sodium azide. Standard proteins (Sigma calibration kit) that include ribonuclease (13.7 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa) and dextran blue (2000 kDa) were used to calibrate the column. Purified samples (about 2 μg) fractionated by native- (Reisfeld, 1962) and SDS–PAGE (Laemmli, 1970) at 15% acrylamide and stained with Coomassie brilliant blue for protein or PAS reagent (Kapitany and Zebrowski, 1973) for glycoprotein. Mid-range standard proteins (Promega) were used in the SDS–PAGE to estimate the subunit size. To determine isoelectric point, about 2 μg pure protein samples in 15–20 μl acetate buffer (pH 5.2) and 3.5 μg/20 μl standard proteins (p*I* values in the 3.6–9.3 range, Sigma) were loaded onto the cathode end of the Ampholine® PAGplate (pH 3.5–9.5) gel. Phosphoric acid (1 M) and sodium hydroxide (1 M) used as the anode and cathode buffers, respectively. Isoelectric focusing performed on a Pharmacia's MULTIPHOR II Electrophoresis Unit at 4 °C and run for 90 min with 25 mA constant current and power supply at 1.5 kV.

For N-terminal amino acid sequencing analysis, 2 μg of purified α-gal 2 was separated on SDS–PAGE and electroblotted onto a polyvinylidene diflouride (PVDF) membrane (Millipore) as described by Matsudaira (1987). The protein band was cut out using a sterilised blade, rinsed in deionised water for 5–10 min, air dried and stored at −20 °C. N-terminal sequencing performed at Microchemical Facility, Institute of Animal Physiology and Genetics, Cambridge, UK and analysed using BLAST search (CNBI) and MultAlin and BoxShade server programmes. The procedures for determination of optimal pH and temperature, heat stability, effects of cations, sugars and concentrations of PNPG, and substrate specificity studies were as previously described (Ali et al., 1998).

3.6. Antibody production and Western blotting analysis

Polyclonal antibody was raised against purified α-gal 2 according to the procedures described earlier (Ali et al., 1998). The IgG1 fraction was separated by a combination of ammonium sulphate precipitation (0–33%) and anion exchange chromatography using a DEAE-Sephacel (Pharmacia) column (Dunbar and Schwoebel, 1990). Crude extracts from various developmental stages of fruits were concentrated via precipitation at 85% ammonium sulphate saturation and dialysed against the extraction buffer for 20–22 h. Extractable proteins (100 μg) fractionated on native-PAGE and electroblotted to a nitrocellulose membrane (Towbin et al., 1979). The α-gal 2 proteins was detected by the ECL (enhanced chemiluminescence) western blotting method according to the manufacturer's instructions (Amersham, Buckinghamshire, UK) using the IgG1 fraction of the antibody (1:500 dilution). Intensity of the bands was analysed with the Bio-Rad Imaging Densitometer and calibrated with known amount of purified α-gal 2 for quantification of the protein level. The preimmune serum was tested and none of the proteins in the extracts immunoreactive.

3.7. In vitro cell wall modification studies

Alcohol insoluble solid (AIS) was prepared from unripe and ripe papaya (Lazan et al., 1995) and carambola fruits (Chin et al., 1999). For in vitro studies, approximately 5 nkat α-gal 2, obtained from fractions containing highest activity peak (Fig. 2(d)), was added to a reaction mixture that contained 15 mg AIS of unripe fruit, 2.14 ml 80 mM sodium acetate (pH 5.0), 0.86 ml BSA (0.1%) and 1–2 drops of toluene. For the papaya fruit, effect of the presence of 10 mM disodium EDTA in the solution also investigated. The mixture incubated for 72 h at 37 °C and the reaction stopped with a brief (3 min) boiling treatment. α-Galactosidase 2 boiled for 15 min, used as control. The supernatant was recovered by centrifugation at 2000*g* for 30 min and assayed for uronic acid content (Filisetti-Cozzi and Carpita, 1991). Gel filtration analysis of the cell wall polyuronides (~1 mg uronic acid) carried out (Lazan et al., 1995) using a Sephacryl S-500 HR (Pharmacia) column (1.6 cm × 60 cm) at a flow rate of 9.5 ml h^{-1} and fractions (2.0 ml) estimated for their uronic acid content.

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