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β-GALACTOSIDASE AND ITS SIGNIFICANCE IN RIPENING MANGO FRUIT

ZAINON MOHD ALI, SANTHI ARMUGAM and HAMID LAZAN*

Departments of Biochemistry and *Botany, Faculty of Life Sciences, Universiti Kebangsaan Malaysia, Bangi, 43600 Selangor, Malaysia

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Key Word Index—*Mangifera indica*; Anacardiaceae; mango fruit; ripening; softening; β-galactosidase; pectin degradation.

Abstract—The fruit extracts of ripening cv. Harumanis mango contained a number of glycosidases and glycanases. Among the glycosidases, β-D-galactosidase (EC 3.2.1.23) appeared to be the most significant. The enzyme activity increased in parallel with increase in tissue softness during ripening. Mango β-galactosidase was fractionated into three isoforms, viz. β-galactosidase I, II and III by a combination of chromatographic procedures on DEAE-Sepharose CL-6B, CM-Sepharose and Sephacryl S-200 columns. Apparent K_m values for the respective β-galactosidase isoforms for *p*-nitrophenyl β-D-galactoside were 3.7, 3.3 and 2.7 mM, and their V_{max} values were 209, 1024 and 62 nkat mg^{-1} protein. Optimum activity occurred at *ca* pH 3.2 for β-galactosidase I and II, and pH 3.6 for β-galactosidase III. Mango β-galactosidase and its isoforms have galactanase activity, and the activity of the latter in the crude extracts generally increased during ripening. The close correlation between changes in β-galactosidase activity, tissue softness, and increased pectin solubility and degradation suggests that β-galactosidase might play an important role in cell wall pectin modification and softening of mango fruit during ripening.

INTRODUCTION

Fruit softening during ripening is mainly a result of the modification of cell wall carbohydrates by the action of wall-associated enzymes [1]. In mango, the activity of the cell wall enzymes, polygalacturonase (PG) (EC 3.2.1.15) and cellulase (EC 3.2.1.4), increases with ripening [2–4]. In addition, β-galactosidase activity also increases [5] and this increase is associated with increased solubility and depolymerization of wall pectins [6]. Evidence further indicates that hemicellulose modification occurs during mango fruit ripening [7]. In spite of the rapid and extensive softening that characterized ripening of many mango cultivars, the significance of the wall-associated enzymes to fruit softening is still unclear.

In mango and in many other tropical fruits, PG activity is low compared with that in tomato [8]. Mango PG appears to occur predominantly as an exo-acting enzyme. The presence of exo-PG in relatively low amounts [8], the lack of relationship between the extent of softening in different cultivars and their PG levels [3, 4], and the rather extensive depolymerization of wall pectins that accompanies ripening [6] all suggest that other enzymes also play important roles in cell wall modification and softening of the fruit.

Evidence is accumulating that β-galactosidase might contribute significantly to fruit softening and cell wall modification [9, 10]. The enzyme also features prominently in accelerated softening of chill-injured fruits [11] or in fruits where softening was retarded by modified atmosphere packaging or coating [12, 13]. β-Galactosidase occurs in a number of isoforms, and the different isoforms may be distributed differentially as related to tissue position, and their activity changed differently during ripening [10, 14, 15]. The objective of this paper is to investigate the significance of mango glycosidases, notably β-galactosidase and its isoforms, in mango fruit softening during ripening.

RESULTS AND DISCUSSION

Glycosidases and pectin modification in ripening mango

The fruit extracts of cv. Harumanis mango contained a number of glycosidases (Fig. 1). With the exception of α-D-glucosidase (EC 3.2.1.20) and β-D-xylosidase (EC 3.2.1.37), the activities of β-D-glucosidase (EC 3.2.1.21), α-L-arabinosidase (EC 3.2.1.55), α-D-mannosidase (EC 3.2.1.24), α-D-galactosidase (EC 3.2.1.22) and β-D-galactosidase (EC 3.2.1.23) were detectable in ripening fruits (Fig. 1). Among the glycosidases, β-galactosidase appeared to be the predominant enzyme. The enzyme activity increased in close correlation with increase in tissue softness or decrease in firmness (Fig. 1a). Extractable protein level also increased (Fig. 2), perhaps suggesting that particular proteins were being accumulated during ripening of the fruit. Our initial evidence

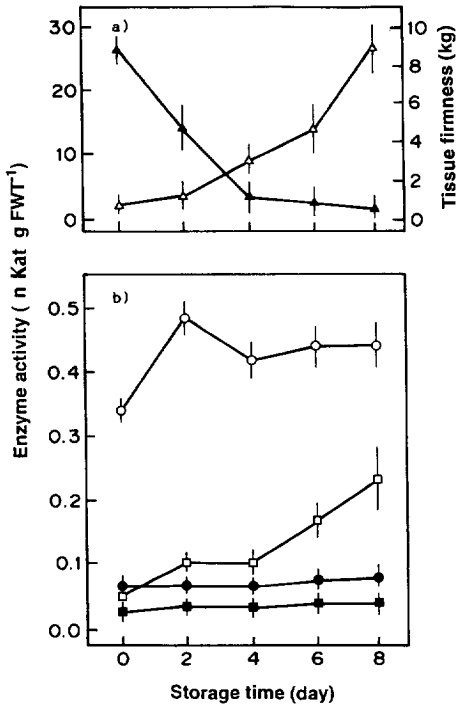

Fig. 1. Changes in tissue firmness and glycosidase activities (a and b) in ripening mango. Tissue firmness, (▲); β-galactosidase, (△); α-galactosidase, (○); α-mannosidase, (□); β-glucosidase, (●); α-arabinosidase, (■). Vertical bars represent s.e.

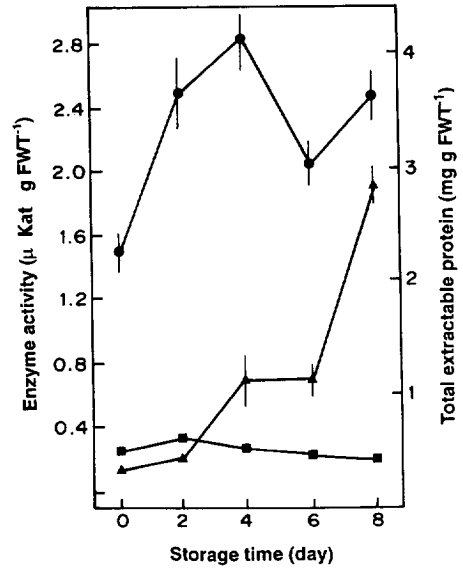

Fig. 2. Changes in extractable protein (▲), galactanase (●) and xylanase (■) activity in ripening mango. Vertical bars represent s.e.

using an indirect, competitive ELISA method seemed to indicate that β-galactosidase immunogenic protein levels increased during ripening [6]. α-Galactosidase activity was moderately high as compared with other glycosidases and the level increased slightly during ripening (Fig. 1b). The activities of α-mannosidase, α-arabinosidase and β-glucosidase in the fruit extracts were low (Fig. 1b). α-Arabinosidase and β-glucosidase activities remained unchanged, whilst that of α-mannosidase increased as the fruits softened. The mango fruit extracts also contained galactanase and xylanase (Fig. 2). The galactanase activity increased to a peak and then decreased, whereas that of xylanase (EC 3.2.1.8) remained unchanged throughout ripening.

Ripening of cv. Harumanis mango was also characterized by depolymerization of cell wall pectins (Fig. 3). Firm, mature fruits (day 0 storage) exhibited a distinct uronic acid peak at the void volume of Sephacryl S-400 column. At day 8, when the fruits were ripe and soft, the peak of the larger molecular size polymers was reduced, and in addition, a new peak at a higher elution volume appeared. As the fruits ripened, their soluble pectins level increased from *ca* 30 to 170 mg g^{-1} ethanol-insoluble residue. The observed increased solubility and depolymerization of wall pectins (Fig. 3) were consistent with those reported earlier [6]. Hemicelluloses were also modified during ripening [7]. Structural and chemical changes that occur in the cell wall of ripening mangoes (Fig. 3) [7, 16] are indicative of the contributory roles of wall enzymes to fruit softening. Decrease in starch content may further contribute to fruit softening during ripening. Of the cell wall hydrolases that were detected in ripening mangoes, PG and β-galactosidase appeared significant [8]. The activity of the enzymes was positively correlated with mango fruit softening with respect to both ripening and depth of the mesocarp tissues [5]. Retarding softening by storage of mango either under modified atmosphere [17] or at low temperature (Nasrijah, Ali and Lazan, unpublished data) retarded the increase in PG and β-galactosidase activity. PG and β-galactosidase might affect pectin modification, whilst cellulase, which also increases in activity during ripening [4], might contribute to hemicellulose modification.

Besides, cellulase, xylanase and mannosidase might also play important roles in hemicellulose modification. Xylanase activity, although it did not increase (Fig. 2),

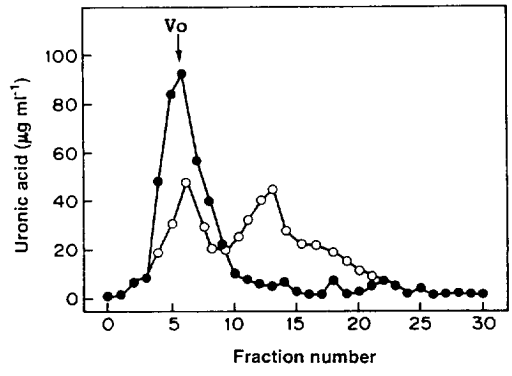

Fig. 3. Gel filtration profiles on Sephacryl S-400 of soluble pectins from day 0 (●) and day 8 (○) mango fruits.

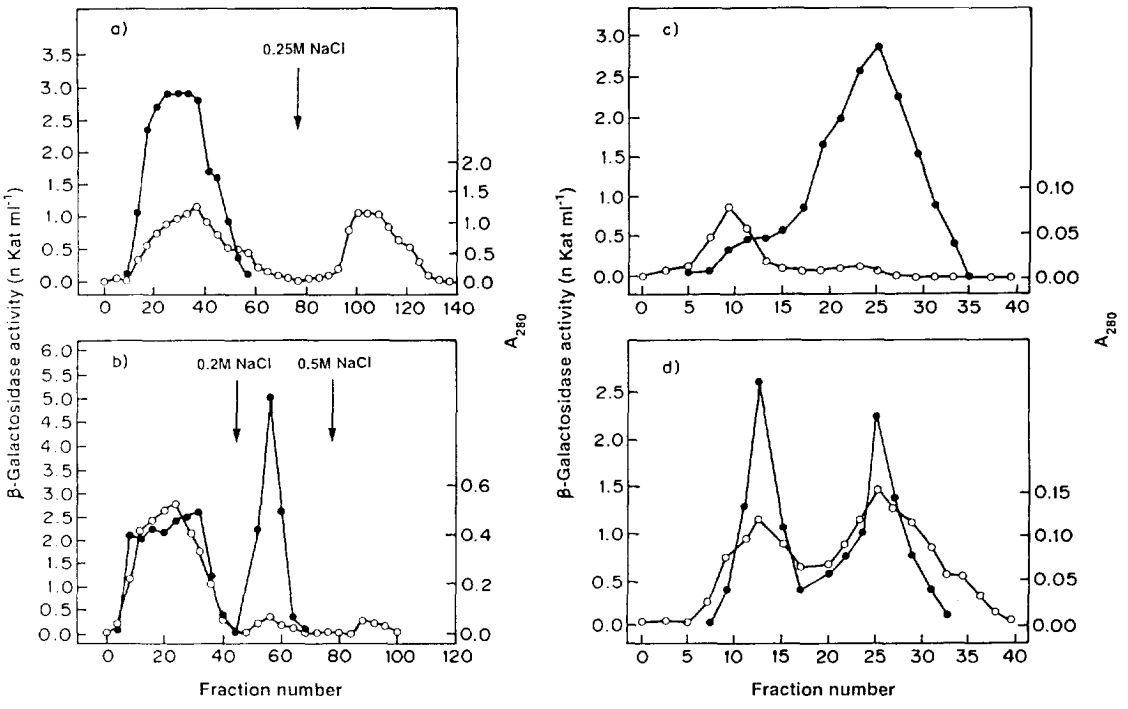

Fig. 4. Elution profile of β-galactosidase activity (●) and A280 (○) on (a) DEAE–Sepharose CL-6B, (b) CM–Sepharose fast flow, (c) 1st Sephacryl S-200 for β-galactosidase I and (d) Sephacryl S-200 for β-galactosidase II and III.

remained relatively high throughout ripening of cv. Harumanis. This may be relevant to the observation that in certain mango cultivars, cell wall xylosyl residues increased as hemicelluloses were being modified during ripening [7]. The role of mannosidase is unclear. Although the enzyme activity increased (Fig. 1), changes in mannosyl residues in the cell wall of ripening mangoes were insignificant [7].

Multiple forms of mango β-galactosidase

β-Galactosidase from ripe mango fruits may exist in at least three isoforms, viz. β-galactosidase, I, II and III (Fig. 4). β-Galactosidase I appeared to be the predominant form. In the initial steps prior to enzyme fractionation, it was found that, occasionally, the supernatant from the crude extracts upon standing, and, on dialysis, will become very viscous and would then be unable to be used in subsequent chromatographic steps. By increasing the pH of the extracting buffer from 4.6 (0.1 M NaOAc containing 1 M NaCl) to 7.6 (0.05 M Tris–HCl containing EDTA), the sample viscosity improved without affecting the extractability of the enzyme. Separation of β-galactosidase multiforms involved the use of anion exchange chromatography on a DEAE–Sepharose column at pH 7.6 in the first step. Although this step was unable to separate the β-galactosidases, almost half of the proteins which were bound to the column were removed (Fig. 4a). In the next step, β-galactosidase activity was resolved into two fractions when cation exchange chromatography on a CM–Sepharose column at pH 5.2 was employed (Fig. 4b). The bound β-galactosidase fraction was loaded onto a Sephacryl S-200 column and eluted as a single peak and named β-galactosidase I (Fig. 4c). The unbound fraction, when loaded onto the same column separately, was resolved into two peaks named β-galactosidase II and β-galactosidase III, respectively (Fig. 4d). The β-galactosidase I was re-run on the same column to remove residual protein contaminants and this preparation appeared as a single protein band on a native PAGE.

Apparent K_m and V_{max} values for β-galactosidase I, II and III were determined from Lineweaver–Burk plots. The K_m values for β-galactosidase I, II and III with *p*-nitrophenyl β-D-galactopyranoside as substrate were 3.7, 3.3 and 2.7 mM, respectively, and the V_{max} values were 209, 1024 and 62 nkat mg^{-1} protein, respectively. The pH optima for the enzymes were between 2.8 and 3.6 for β-galactosidase I and II, and pH 3.2–4.0 for β-galactosidase III. However, crude enzyme appeared to react optimally at pH 4.0–4.2. These apparent K_m, V_{max} and pH optimum values are generally similar to the values for β-galactosidase from other fruits [9, 14, 15, 18, 19]. Heat stability studies showed that the 50% loss in enzyme activity occurred at 53°, 54.5° and 57.5° for β-galactosidase I, II and III, respectively. Addition of salts (0.15 to 0.75 M for NaCl and KCl, and 0.15 to 0.75 mM for $CaCl_2$) to the reaction mixtures generally have no effect on the β-galactosidases activities, except that in the presence of 0.45–0.60 mM $CaCl_2$, β-galactosidase I activity was stimulated slightly to *ca* 135%, and in the presence of

Table 1. Substrate specificity of β-galactosidase

Substrate	Enzyme activity(nkat mg^{-1} protein)		
	β-Gal I	β-Gal II	β-Gal III
p-Nitrophenyl β-D-galactopyranoside	307	500	238
p-Nitrophenyl β-D-glucopyranoside	N.D.	N.D.	N.D.
p-Nitrophenyl α-D-mannopyranoside	N.D.	N.D.	N.D.
p-Nitrophenyl α-L-arabinopyranoside	N.D.	N.D.	N.D.
Xylan	N.D.	N.D.	N.D.
Galactan	953	3610	383

N.D. = Not detectable.

0.75 M KCl, β-galactosidase II activity was reduced to *ca* 75%. At a similar concentration range, papaya fruit β-galactosidases activities were unaffected by these cations [15]. Addition of much lower concentrations (0.5–1 mM) of the same cations also had no effect on β-galactosidase from carrot cell cultures [20].

Substrate specificity study (Table 1) showed that the β-galactosidase isoforms were able to degrade galactan but not xylan or other *p*-nitrophenylglycoside substrates, indicating that the enzyme preparations were free from such glycosidases as β-glucosidase, α-mannosidase and α-arabinosidase. β-Galactosidase preparation from coffee berry [19] and the three β-galactosidase isoforms from ripe papaya fruits [15] also have the ability to hydrolyse galactan. However, in tomato, only β-galactosidase II was found to have galactanase activity [14]. Besides papaya and tomato, multiple forms of β-galactosidase have also been reported in ripening avocado [10].

The significance of β*-galactosidase to mango fruit softening*

That mango β-galactosidase is probably one of the key enzymes in pectin modification, perhaps complementing that of PGs, is supported by indirect evidence. The rather extensive depolymerization of wall pectins (Fig. 3) was inconsistent with the observation that exo-PG predominates in ripening mango [8]. In addition, mango contains a low PG-activity which was *ca* 0.0125 times as much as that in tomato fruits. β-Galactosidase might act as a pectin debranching enzyme, and may thus contribute to pectin modification. The ability of β-galactosidase purified from muskmelon [9], avocado [10] and papaya (Lazan and Ali, unpublished data) fruits to degrade wall pectin is consistent with the idea that β-galactosidase is possibly a pectin debranching enzyme. In mango, loss of galactosyl, arabinosyl and rhamnosyl residues from the cell wall during ripening has been reported [7, 16]. Such losses of neutral sugars could possibly be attributed to hydrolysis of galactans and arabinogalactans by β-galactosidase having galactanase activity. Arabinogalactans and galactans are believed to be the neutral sugar side-chain polymers linking rhamnogalacturonan backbone of pectins to the hemicelluloses [21]. Mango β-galactosidase and its isoforms, β-galactosidase I, II and III (Figs 1 and 4), were found to have galactanase activity (Table 1), and the activity of the latter (Fig. 2) generally increases during ripening. Preliminary studies [6] indicate that β-galactosidase immunogenic protein levels increased in parallel with increase in total β-galactosidase activity, again suggesting the importance of the enzyme in mango fruit softening during ripening.

EXPERIMENTAL

Plant material. Harvest maturity mango (*Mangifera indica* cv. Harumanis) fruits were purchased from the State of Perlis Agriculture Department, Malaysia, and treated as described previously [3]. The fruits were left to ripen at ambient temp. (24–28°). Sampling of 5 fruits was carried out every alternate day (i.e. day 0, 2, 4, 6 and 8 of storage) and the fruits were peeled, cut into 1 cm^3 cubes, and kept separately at − 80° until used.

Tissue firmness. Firmness estimation of each fruit prior to tissue sampling was carried out by using a McCormick Pressure Tester according to the procedure described in ref. [5].

Extraction of soluble pectins. Soluble pectins were extracted from EtOH-insoluble residues by the method described previously for papaya [22]. This method was adopted for routine analysis because it was observed that pectins extracted from these residues gave comparable amounts and an identical profile on gel filtration to those extracted from similar residues that have been previously treated with DMSO to remove starch.

Analysis of pectin depolymerization. Pectin depolymerization was analysed using a Sephacryl S-400 column (1.6 × 46 cm). *Ca* 0.8–1 mg soluble pectins (galacturonic acid equivalents) in 30 mM NaOAc pH 5 containing 10 mM EDTA was applied to the column which had been equilibrated with the same buffer. Frs (2 ml) were collected at a flow rate of 18 ml hr^{-1} and the uronide level in each fr. was determined [23].

Extraction and assay of glycosidases and glycanases. Extraction of enzymes was carried out as previously described [3]. Tissue (10 g) was homogenized in 20 ml 0.1 M Na-citrate, pH 4.6 containing 1 M NaCl, 0.5% (w/v) soluble PVP-40 and 10 mM 2-mercaptoethanol. The homogenate was left for 30 min with occasional stirring, centrifuged at 17 000 *g* for 30 min and the supernatant was recovered for enzyme assay. Glycosidases

were assayed according to ref. [5] with some modifications. The assay mixt. consisted of 0.52 ml 0.1 M Na-citrate of optimum pH for each enzyme (pH 5.2 for α-arabinosidase, α-mannosidase and β-glucosidase; pH 4.7 for α-galactosidase and pH 4.1 for β-galactosidase), 0.4 ml 0.1% (w/v) BSA and 0.4 ml 13 mM of the corresponding *p*-nitrophenyl derivatives of α-L-arabinopyranoside, α-D-mannopyranoside, β-D-glucopyranoside, α-D-galactopyranoside and β-D-galactopyranoside (Sigma) as substrates. The reaction mixt. was incubated at 37° for 10 min before addition of 0.08 ml sample. The enzymes were assayed for 15 min at the same temp. Na_2CO_3 (2 ml of 0.2 M) was added to stop the reaction and the *p*-nitrophenol formed was determined from the absorbance at 415 nm. All glycosidase activities were expressed as nmol *p*-nitrophenol formed per sec (nkat) per g fr. wt. Samples for glycanase assays were desalted using a 10 ml Sephadex G-25 (Pharmacia) column. For galactanase, the assay mixts which consisted of 0.36 ml 0.1 M NaOAc pH 5.4, 0.1 ml 1% galactan (Aldrich), 0.25 ml H_2O and 0.04 ml sample were incubated for 30 min at 45°, whereas for xylanase, assay mixtures made up of 0.525 ml 0.1 M NaOAc pH 5, 0.15 ml 2% xylan (Sigma) and 0.075 ml sample were incubated for 1 hr at 45°. The reducing groups released from both galactan and xylan were determined using the DNS reagent at *A* 550 nm. In all assays boiled enzymes were used as blanks.

Purification of β-*galactosidase.* Tissues (100 g) from day 8 fruits were extracted in 200 ml 0.05 M Tris–HCl pH 7.6 containing 13 mM EDTA, 0.5% PVP-40 and 10 mM 2-mercaptoethanol as described above. The supernatant was dialysed against 0.05 M Tris–HCl pH 7.6 containing 1 mM DTT overnight with a change of fresh buffer after the first 4 hr. The sample was loaded onto a DEAE–Sepharose CL 6B (Pharmacia) column (5.0 × 8.5 cm) which had been equilibrated with the same buffer at 40 ml hr^{-1}, and 6.8 ml frs were collected. Most of the β-galactosidase activity was not adsorbed by the column. The pooled frs of the unadsorbed β-galactosidase were dialysed overnight against 0.1 M NaOAc pH 5.2 containing 1 mM DTT and passed through a CM–Sepharose (fast flow; Pharmacia) column (2.5 × 16.5 cm) previously equilibrated with the same buffer at a flow rate of 35 ml hr^{-1} and frs of 6.2 ml were collected.

There were two peaks of β-galactosidase activity; one sharp peak adsorbed by the cation exchange column was eluted in 0.2 M NaCl (β-galactosidase I), and the second peak was coming through with the starting buffer. High specific activity of β-galactosidase I frs were pooled, pptd at 80% $(NH_4)_2SO_4$ satn, dissolved in min. vol. of 0.1 M NaOAc, pH 5.2 containing 1 mM DTT and loaded onto a Sephacryl S-200 superfine (Pharmacia) column (2.5 × 51 cm) previously equilibrated with the same buffer. The flow rate was 25 ml hr^{-1} and frs vol. was 6.2 ml. The pooled frs of β-galactosidase I were re-run on the same column to remove further protein contaminant. The unabsorbed β-galactosidase frs from CM–Sepharose column were pooled separately, treated as above and loaded onto the same Sephacryl S-200 column previously equilibrated with the same buffer. β-Galactosidase activity was resolved into two peaks and named β-galactosidase II and β-galactosidase III, respectively.

Protein determination. Protein concn was determined according to the method of ref. [24] with BSA as a standard. Samples from crude extracts were pptd with 10% TCA, rinsed with 5% TCA, and subsequently dissolved in 0.1 M NaOH prior to protein determination.

Polyacrylamide gel electrophoresis. The purity of β-galactosidase isoforms was examined by native PAGE [25] using 10% acrylamide for the separating gel. Coomassie Brilliant Blue R was used for staining.

Effect of pH on β-*galactosidase activity.* The pH optimum of the β-galactosidase isoforms were determined by assaying the enzymes in the presence of 0.52 ml 0.1 M Na-citrate at pH ranged from 2 to 6.

Effect of temperature on β-*galactosidase stability.* β-Galactosidase isoforms (0.50–0.55 μg protein) were exposed to a range of temps (4–65°) for 10 min prior to enzyme assay and the residual activities were measured.

Effect of cations on β-*galactosidase activity.* The effects of cations on the activity of β-galactosidase isoforms were examined by adding KCl or NaCl (final concns of 0.15 to 0.75 M) or $CaCl_2$ (final concns of 0.15 to 0.75 mM) to the assay mixtures.

K_m *and* V_{max} *values of* β-*galactosidase.* Each isoform was assayed in the presence of 2–16 mM *p*-nitrophenyl β-D-galactopyranoside and the values of K_m and V_{max} were determined from the Lineweaver–Burk plots.

Substrate specificity. For substrate specificity studies, β-galactosidase I, II and III frs were assayed for other glycosidase and glycanase activities using 13 mM *p*-nitrophenyl derivatives of β-D-glucopyranoside, α-D-mannopyranoside or α-L-arabinopyranoside or 1% (w/v) galactan or 2% (w/v) xylan as substrates. The procedures were as detailed for the respective enzymes assays in crude extracts.

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REFERENCES

1. Huber, D. J. (1983) *Hort. Rev.* **5**, 169.
2. Roe, B. and Bruemmer, J. H. (1981) *J. Food Sci.* **46**, 186.
3. Lazan, H., Ali, Z. M., Wah, L. K., Voon, J. and Chaplin, G. R. (1986) *ASEAN Food J.* **2**, 93.
4. Abu-Sarra', A. F. and Abu-Goukh, A. A. (1992) *J. Hortic. Sci.* **67**, 561.
5. Lazan, H., Ali, Z. M., Soh, J. S. and Talkah, Z. (1993) *Acta Hortic.* **341**, 500.
6. Ali, Z. M., Lazan, H., Tee, L. H. and Nasrijal, N. H. (1993) *Proc. IRPA-UKM Workshop* **2**, 385.
7. Mitcham, E. J. and McDonald, R. E. (1992) *J. Am. Soc. Hort. Sci.* **117**, 919.
8. Lazan, H. and Ali, Z. M. (1993) *ASEAN Food J.* **8**, 47.
9. Ranwala, A. P., Suematsu, C. and Masuda, H. (1992) *Plant Physiol.* **100**, 1318.

10. Ian De Veau, E. J., Gross, K. C., Huber, D. J. and Watada, A. E. (1993) *Physiol. Plant.* **87**, 279.
11. Ali, Z. M., Lazan, H., Ishak, S. N. and Selamat, K. (1993) *Acta Hortic.* **343**, 230.
12. Lazan, H., Ali, Z. M. and Selamat, K. (1993) *Acta Hortic.* **343**, 141.
13. Selamat, K. (1993) M.Sc. Thesis. Universiti Kebangsaan, Malaysia.
14. Pressey, R. (1983) *Plant Physiol.* **71**, 132.
15. Goh, L. Y. (1993) M.Sc. Thesis. Universiti Kebangsaan, Malaysia.
16. Brinson, K., Dey, P. M., John, M. A. and Pridham, J. B. (1988) *Phytochemistry* **27**, 719.
17. Lazan, H., Ali, Z. M. and Sim, W. C. (1990) *Acta Hortic.* **269**, 345.
18. Bartley, I. M. (1974) *Phytochemistry* **13**, 2107.
19. Golden, K. D., John, M. A. and Kean, E. A. (1993) *Phytochemistry* **34**, 355.
20. Konno, H., Yamasaki, Y. and Katoh, K. (1986) *Plant Physiol.* **68**, 46.
21. Talmadge, K. W., Keegstra, K., Bauer, W. D. and Albersheim, P. (1973) *Plant Physiol.* **51**, 158.
22. Lazan, H., Ali, Z. M., Liang, K. S. and Yee, K. L. (1989) *Physiol Plant.* **77**, 93.
23. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484.
24. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.
25. Reisfeld, R. A., Lewis, U. J. and William, D. E. (1962) *Nature* **195**, 281.