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Characteristics of an α-galactosidase associated with grape flesh

Han-Chul Kang[a,*], Seon-Hwa Lee[b]

[a]*Department of Plant Nutrition, National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon 441-707, South Korea*

[b]*National Horticultural Research Institute, Rural Development Administration, Suwon 440-310, South Korea*

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Abstract

α-Galactosidase activity in grape flesh (*Vitis venifera* L. Muscat of Alexandria) was characterized by a marked increase in its activity 4 weeks after fruit bearing. After 12 weeks the specific activity of the enzyme had increased 15-fold. Several other glycosidases were measured at different stages of fruit development but none showed the increased levels of activity displayed by this α-galactosidase. α-Galactosidase activity (unit/g·fresh wt) increased by 52% during postharvest storage, whereas the unripe grape showed a "stagnancy" for 10–15 days prior to the increase. An α-galactosidase was partially purified ca. 103-fold from grape flesh of *Vitis labruscana* Honey black, by a procedure involving ammonium sulfate fractionation, Biogel P-60, melibiose-agarose, and Sephacryl S-200 chromatographic separations. The enzyme was effectively separated by affinity chromatography on melibiose-agarose, and was a monomer of 40–45 kDa as determined by SDS-PAGE and Sephacryl S-200 chromatographic analysis. The hydrolysis rate of *p*-nitrophenyl-α-D-Gal (PNP-α-D-Gal) was 4.2 times higher than that of PNP-β-D-Gal, implying an apparent α-anomer specificity, and natural oligosaccharides such as melibiose, stachyose, and raffinose were also considerably hydrolyzed. The enzyme was active over a narrow pH range with an optimal hydrolysis of stachyose and PNP-α-D-Gal at pH 6.0 and 7.0, respectively. EDTA or 1,10-phenanthroline did not substantially affect enzyme activity.

Keywords: *Vitis veniferae*; Vitaceae; Grape; α-Galactosidase

1. Introduction

α-Galactosidase (E.C. 3.2.1.22, α-D-galactoside galactohydrolase) is one of the exoglycosidases, capable of hydrolysing α-1,6 linked α-galactoside residues. α-Galactosidases are widely distributed in intra- or extracellular forms in microorganisms, plants and animals. The enzyme in plants, especially, has been greatly investigated from seeds in relation to germination (Dey and Pridam, 1969; Barham et al., 1971; McCleary and Matheson, 1974; Dey et al., 1983; Corchette and Guerra, 1987) and is also found in leaves and other tissues (Gatt and Baker, 1970; Thomas and Webb, 1978; Smart and Pharr, 1980; Gaudreault and Webb, 1983; Burns, 1990). Diverse forms of α-galactosidase occur occasionally in many plants, having molecular weights ranging from 25 to 270 kDa and different properties (Dey and Pridam, 1969, 1972; Thomas and Webb, 1977; Hankins et al., 1980; Smart and Pharr, 1980; Dey et al., 1983; Mujer et al., 1984; Haibath et al., 1991; Chrost and Schmitz, 1997; Irving et al., 1997). α-Galactosidases are generally involved in metabolic utilization of oligosaccharides such as raffinose, stachyose, melibiose, and galactomannan, that are even more widely distributed in storage organs such as seeds, roots, and tubers (McCleary and Matheson, 1974; Kandler and Hopf, 1980). α-Galactosidases have been implicated with the metabolism of galactolipids (Sastry and Kates, 1964; Pridam and Dey, 1974). In addition to these hydrolysis reactions, transglycosylation is catalyzed by the enzyme with somewhat broad acceptor specificity (Dey and Pridam, 1972).

Despite this wide distribution and diversity, α-galactosidases seem to be less abundant in fruits compared with other plant organelles. Biochemical/physiological aspects in fruits differ from those of other tissues or organelles in plants, and hence other types of α-galactosidase may be present in grape. Characterization of α-galactosidase in grape flesh would be helpful in understanding the physiological role of the enzyme. In this

* Corresponding author. Tel.: +82-31-290-0260; fax: +82-31-290-0261.

E-mail address: hckang3436@hanmail.net (H.-C. Kang).

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paper, we partially purified an α-galactosidase associated with grape flesh and present some of its biochemical characteristics.

2. Results and discussion

2.1. α-Galactosidase activity from grape flesh

The glycosidase activities of flesh (mesocarp plus placental tissue) from *Vitis venifera* L. Muscat of Alexandria are shown in Table 1. Of the various glycosidases assayed, α-galactosidase was the most active, followed by α-mannosidase, as measured by the assays at pH 5.0 or 7.0. Significant activities of α- and β-glucosidase, and β-galactosidase were found at acidic or neutral pH. Glycosidases were also evaluated from the flesh of other grape cultivars, Honey black, Marguerite, Edelweiss, Agawan, Egiodola, Green hungarian, and Takasumi at pH 7.0. For all of the strains tested, α-galactosidase was the most active, although other glycosidases also showed significant activities (data not shown). Fig. 1 shows the variation in the specific activities of galactosidase, glucosidase, and α-mannosidase in flesh from Muscat of Alexandria at different stages of fruit development. These glycosidase activities were scarcely found at the unripe stage (approximately up to 4 weeks from fruit bearing). After this stage, the activities increased rapidly to different extents. Approximately 15 times increase in α-galactosidase activity was observed during the ripening stage from 4 to 12 weeks. The most prominent increase was found at the mid-term of ripening stage (4–8 weeks from fruit bearing). The expression of α-galactosidase in the flesh was characterised by an increase in activity per g·fresh wt as well as in the activity per mg·protein along with the ripening stage. These results suggest that α-galactosidase might play an important role in grape flesh during fruit development. The marked expression of α-galactosidase synchronizing with the ripening stage could be correlated with the report of Gross (1986) that applied galactose stimulated ethylene production and promoted ripening of mature green tomatoes. But the results presented here do not elucidate any role for the α-galactosidase in the grape flesh. Putative variation of α-galacto sidase during postharvest storage at 4°C was assessed from the flesh of Honey black harvested after 9 or 12 weeks from fruit bearing (Fig. 2). In the latter grape, the α-galactosidase activity (unit/g· flesh wt) increased by 52% of control after 15 days of storage and then declined slowly. The unripe grapes harvested after 9 weeks, however, showed a "stagnancy" for 10–15 days of storage before an increase in activity occurred, and then the activity slowly increased albeit to a lesser extent than the more ripe grape.

Table 1
Distribution of glycosidases in grape flesh (*Vitis venifera* L. Muscat of Alexandria). The activity was measured for the grape harvested after 3 months from fruit bearing. The enzyme assay was run by hydrolysis of PNP-glycosides (2 mM) in 50 mM Na-acetate (pH 5.0) or K-phosphate (pH 7.0) containing 0.5% glycerol. Glycosidase activities were represented as units per g·fresh wt. Soluble solids content of the grape berry was 17.1°Bx. Activity values were expressed as means ± S.E. of three independent experiments

Substrate	Glycosidase activity (unit/g·flesh wt)	
	pH 5.0	pH 7.0
PNP-α-D-Gal	545.6±26.5	814.2±31.6
PNP-β-D-Gal	206.4±18.4	190.8± 9.5
PNP-α-D-Glc	195.4±12.0	374.1±11.7
PNP-β-D-Glc	201.9±10.2	309.0±22.1
PNP-α-L-Fuc	49.2± 4.3	101.4± 2.5
PNP-β-D-Fuc	31.3± 2.5	55.4± 3.1
PNP-α-D-Man	398.3±22.3	569.8±18.4
PNP-α-L-Ara	8.9± 0.2	6.1± 0.6
PNP-α-L-Rha	0.6± 0.1	0.8± 0.2
PNP-β-D-GlcUA	0.4± 0.1	0.5± 0.1
PNP-α-D-GlcNAc	2.4± 0.2	0.9± 0.2
PNP-β-D-GlcNAc	1.6± 0.1	0.7± 0.1
PNP-β-D-GalNAc	57.4± 2.4	63.3± 4.5
Polygalacturonic acid[a]	5.9± 0.5	7.7± 0.2

[a] The polygalacturonase activity was measured using 2-cyanoacetamide (Gross, 1982).

2.2. Purification of α-galactosidase

Purification of α-galactosidase was performed using flesh of *Vitis labruscana* Honey black. With regard to

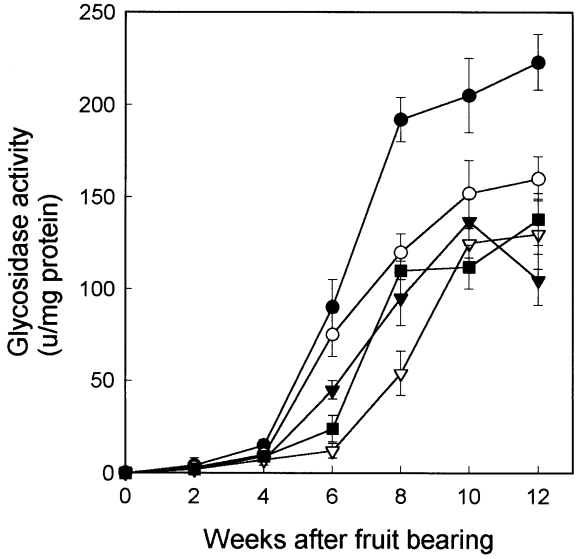

Fig. 1. Occurrence of glycosidases at different stages of fruit development. Glycosidase activities in grape flesh (*Vitis venifera* L. Muscat of Alexandria) was assessed up to 3 months after fruit bearing. α-Galactosidase (-●-), β-galactosidase (-▽-), α-mannosidase (-○-), α-glucosidase (-■-), and β-glucosidase (-▼-) activities were measured using PNP-glycosides and represented as unit per mg·protein. Activities per g·fresh wt increased also gradually along with the ripening stage (These data are not represented in the figure). Each value was the average of three replicate assays.

the α-galactosidase extraction from grape flesh, we note that homogenization of grape flesh with 0.2 M Na_2CO_3 resulted in an increase in α-galactosidase activity (unit/g·fresh wt) by 3.1 times compared with the extraction without Na_2CO_3. Alternatively, addition of 0.05 N NaOH caused the enzyme activity to increase as much as 2.8 times. Thus, the increase of ionic strength and/or the augmentation of pH (pH was elevated to 5.1 from 3.9.) appeared to release more α-galactosidase from the grape flesh. The α-galactosidase from grape flesh was stable during the initial steps of the purification procedures. The stability gradually decreased as the purification steps proceeded and dilution of the enzyme also accelerated a decrease in activity (data not shown). Stabilization or restoration of activity was attempted by adding dithiothreitol (2 and 10 mM) or EDTA (1 and 5 mM), but the stability of α-galactosidase was not affected by these compounds. The activity loss was considerably prevented by addition of glycerol in the equilibration/elution buffers during the purification procedure.

The enzyme was partially purified by salting out with ammonium sulfate and using chromatographic steps involving Biogel P-60, melibiose-agarose, and Sephacryl S-200 columns respectively. A peak of α-galactosidase activity was resolved on melibiose-agarose chromatography.

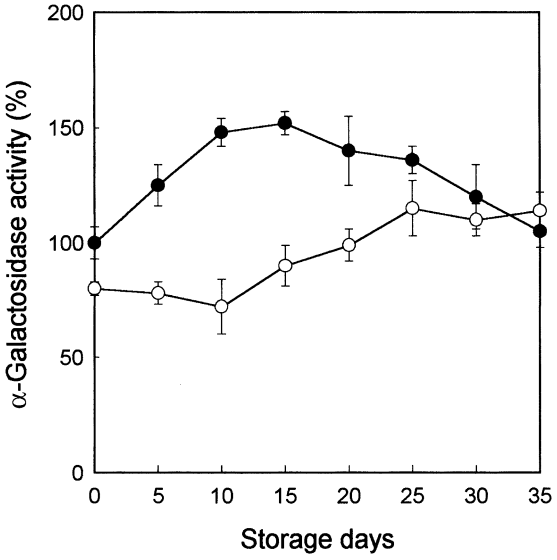

Fig. 2. Activity variation of α-galactosidase during postharvest storage. Grapes (Honey black) were collected after 9 weeks (-○-) and 12 weeks (-●-) and stored at 4°C. The fruits reached each storage day were stored at −70°C. The α-galactosidase activity in flesh was measured at the same time. One hundred per cent activity of α-galactosidase was 109 unit/g·fresh wt. The α-galactosidase activity increased more rapidly at room temperature than at 4°C. Difficulties in storage at room temperature hampered the test for more prolonged intervals (data not shown). Protein concentration was roughly consistent during postharvest storage. Soluble solid contents increased parallel with the storage days as follows: from 12.1 to 14.5 and from 14.7 to 18.1°Bx in grapes harvested after 9 and 12 weeks, respectively. Each value was the average of three replicate assays.

The fractions obtained from the affinity chromatography were further purified using Sephacryl S-200 chromatography (Fig. 3). This gel chromatography was the final step in the purification of α-galactosidase because its instability impeded any further chromatography. Specific activity of the enzyme was increased more than 103-fold by the purification procedure (Table 2). SDS-PAGE analysis of the main fractions with peak activity from the Sephacryl S-200 chromatography showed one major band with a monomeric molecular mass of 45 kDa and some faint bands (Fig. 4). Affinity chromatography on melibiose-agarose was crucial for enzyme purification. Even though melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) might not be perfect as an affinity ligand for chromatography, this carbohydrate seems to be effective for the enzyme purification in

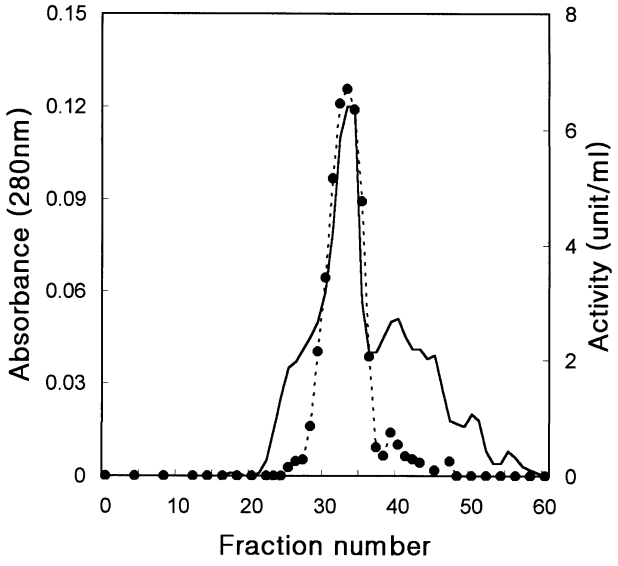

Fig. 3. Elution profile of α-galactosidase from Sephacryl S-200 chromatography. The active fractions from melibiose-agarose chromatography were loaded onto a Sephacryl S-200 column that was equilibrated with K-phosphate buffer (pH 7.0, 50 mM) containing 2% glycerol. Proteins were eluted using the same buffer along with measurement of absorbance at 280 nm (—). The α-galactosidase activity (unit/ml, -●-) was monitored using PNP-α-D-Gal as a substrate. One fraction volume was 5 ml.

Table 2
Purification summary of α-galactosidase from grape flesh of *Vitis labruscana* Honey black[a]

Purification step	Total protein (mg)	Total activity (μ)	Specific activity (μ/mg protein)	Purification fold
Crude extract	980.1	490.1	0.50	1.00
Ammonium sulfate	275.4	294.7	1.07	2.14
Biogel P-60	64.2	201.9	3.14	6.28
Melibiose-agarose	5.8	140.5	24.22	48.44
Sephacryl S-200	1.9	98.0	51.58	103.16

[a] α-Galactosidase assay used 2 mM PNP-α-D-Gal as a substrate.

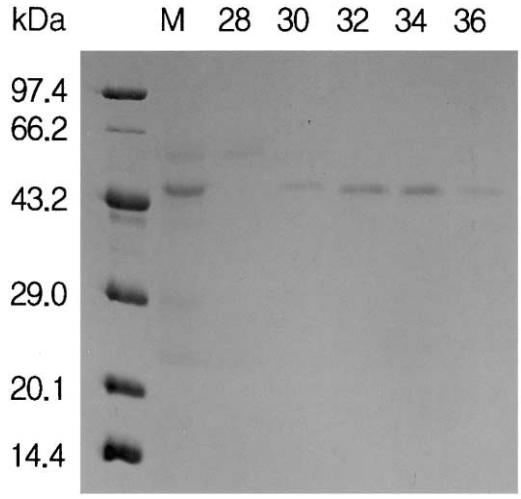

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified α-galactosidase from the grape flesh. Outer left lane: reference proteins consisted of phosphorylase b (M_r; 97,400), BSA (66,200), ovalbumin (43,000), carbonic anhydrase (29,000), trypsin inhibitor (20,100), and lysozyme (14,400). Other lanes: M, the active fraction from melibiose-agarose chromatography; 28–36, the fraction numbers from the Sephacryl S-200 chromatography. The gel was stained with Coomassie Brilliant Blue R-250 and then with silver nitrate. Some faint bands (35 and 38 kDa) were observed in lanes 34 and 36 (not very visible in gel photo).

view of 7.7 times increase in specific activity during the stage from Biogel P-60 to affinity chromatography. The enzyme was stored in 20% glycerol at −70°C, and over 85% of the initial activity was retained after 3 months.

2.3. Substrate specificity of α-galactosidase

The purified α-galactosidase was assayed using different PNP-glycosides (Table 3a). The PNP-α-D-Gal was hydrolyzed more efficiently by the purified galactosidase than any other substrate tested, and *o*-nitrophenyl α-D-Gal (ONP-α-D-Gal) was also easily hydrolyzed. β-D-gal was scarcely hydrolyzed, showing 16% of the α-D-Gal hydrolysis (control), and the extent of 6-*O*-β-D-Gal-β-D-Gal was similarly low. Another galactose-related compounds such as β-D-Lac, Lac-*N*-bioside, and β-D-GalUA were rarely hydrolyzed, resulting in 4.3–12.6% activity of the control, and β-D-GalNAc was not efficiently hydrolyzed. The rapid hydrolysis of α-D-Gal and low hydrolysis of β-D-Gal suggest that the galactosidase is α-anomer specific.

Table 3b shows the hydrolysis of a range of natural or synthetic di-polysaccharides related with galactose. The activity represented as amount of galactose liberated was compared with the hydrolysis of ONP-α-D-Gal (control). ONP-α-D-Gal was used as a control substrate in place of PNP-α-D-Gal since *p*-nitrophenol was an inhibitor of the galactose dehydrogenase used to measure the galactose released (Bhalla and Dalling, 1984). Considerable liberations of galactose were obtained from natural oligosaccharides such as melibiose, stachyose, and raffinose, and the hydrolysis extent from stachyose was the most, showing 32.4% of control. A synthetic disaccharide, 4-*O*-α-D-Gal-D-Gal, was also significantly hydrolyzed. Poly-galacturonic acid was not effectively

Table 3a
Substrate specificity of α-galactosidase assayed using PNP-glycosides. The purified α-galactosidase was assayed for its activity using different PNP-glycosides (2 mM) in K-phosphate (50 mM, pH 7.0) containing 0.5% glycerol. The activities measured by the standard assay method were compared with the hydrolysis of PNP-α-D-Gal (100%), which was 49 units/mg·protein. All values were the mean of two replicate assays

Substrates	Relative activity (%)
PNP-α-D-Gal	100.0
ONP-α-D-Gal[a]	78.2
PNP-β-D-Gal	16.0
PNP-β-D-Lac	4.3
PNP-Lac-*N*-bioside	4.7
PNP-β-D-GalUA	12.6
PNP-6-*O*-β-D-Gal-β-D-Gal	16.1
PNP-α-D-Glc	7.0
PNP-β-D-Glc	6.2
PNP-α-L-Fuc	0
PNP-β-D-Fuc	0
PNP-α-D-Man	3.7
PNP-α-L-Ara	<1.0
PNP-α-L-Rha	0
PNP-β-D-GlcUA	<1.0
PNP-α-D-GlcNAc	<1.0
PNP-β-D-GlcNAc	<1.0
PNP-β-D-GalNAc	<1.0

[a] ONP-α-D-Gal is *o*-nitrophenyl α-D-galactopyranoside.

Table 3b
Substrate specificity of α-galactosidase for natural or synthetic carbohydrates. The purified α-galactosidase was incubated with galactose related carbohydrates (2 mM) in K-phosphate (50 mM, pH 6.0) containing 0.5% glycerol, and estimation of galactose released was performed as described in Experimental. Polygalacturonic acid was used as a substrate for polygalacturonase assay, and the activity determined using 2-cyanoacetamide. The activities were compared with the hydrolysis of ONP-α-D-Gal (100%), which was 42 units/mg · protein. All values were the mean of three replicate assays

Substrates	Free galactose released (%)
ONP-α-D-Gal	100.0
Melibiose	17.1
D-Lactose	7.6
Lactulose	2.0
Lacto-*N*-tetraose	2.7
Lactobionic acid	7.1
Melibionic acid	3.0
3-*O*-β-D-Gal-D-Ara	<1.0
4-*O*-α-D-Gal-D-Gal	26.7
4-*O*-β-D-Gal-D-Man	12.7
4-*O*-β-D-Gal-D-Glc	11.3
Stachyose	32.4
Raffinose	19.8
Polygalacturonic acid	<1.0

hydrolyzed, suggesting that the purified enzyme is not a polygalacturonase. Rapid hydrolysis of the synthetic substrate PNP-α-D-Gal and relatively slow hydrolysis of the oligosaccharides were similar to the α-galactosidase from *Cucurbita pepo* leaves (Thomas and Webb, 1977).

2.4. Other characteristics

Apparent molecular mass of the α-galactosidase was also determined by a gel filtration chromatography. The 34th fraction obtained from the final chromatographic step was co-injected with size marker onto a Sephacryl S-200 column. This protein mixture was eluted along with monitoring of the activity (unit/ml) peak, and the molecular mass was calculated to be ca. 40 kDa. On the analogy of the result from this gel chromatography, the 45 kDa band in the SDS-PAGE (Fig. 4) was considered as an α-galactosidase since 40 kDa of molecular mass determined from the chromatography approached to this value and the intensities of the 45 kDa bands were roughly proportional to the activity profile.

The α-galactosidase was further characterized by measuring its activity in buffers of varying pH using 2 mM PNP-α-D-Gal or stachyose as substrates (Fig. 5-A and B). When PNP-α-D-Gal was used as a substrate, the optimum pH for the activity was estimated to be around 7.0. But the pH profile assayed with a natural substrate, stachyose, resulted in a lowered pH optimum of 6.0. The pH for half maximal activity was ca. 4.5–5.2 and the activity rapidly increased from pH 4.0 to 6.0–7.0. This rapid increase in the activity up to pH 6.0–7.0 could have a correlation with the environment of grape since pH in flesh increases into a weaker acidity as ripening proceeds. Thus, increase in pH during ripening might be an important factor of α-galactosidase regulation in the grape flesh. However, it was not confirmed in this experiment whether such pH variation play an exclusive role in the enzyme activation.

The requirement of divalent cations for the α-galactosidase activity was examined using 1 mM Mg^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, and Ca^{2+} with chloride ion as a counter part ion. Addition of Fe^{2+} ion to the assay mixture in the absence of EDTA elevated the α-galactosidase activity by 46%. Other cations did not significantly increase or decrease the activity, except 49% inhibitory effect by Cd^{2+} (detailed data not shown). 1, 10-Phenanthroline or EDTA (0.5 and 2.0 mM) resulted in only a slight decrease in activity ranging from 90 to 98% of the initial activity (detailed data not shown).

Heat stability was examined with the 36th fraction from the Sephacryl S-200 chromatography. The enzyme was preincubated at 30, 45, or 60°C for 10 min and assayed using PNP-α-D-Gal. The activity decreased to 62% of the initial activity when preincubated at 60°C (detailed data not shown).

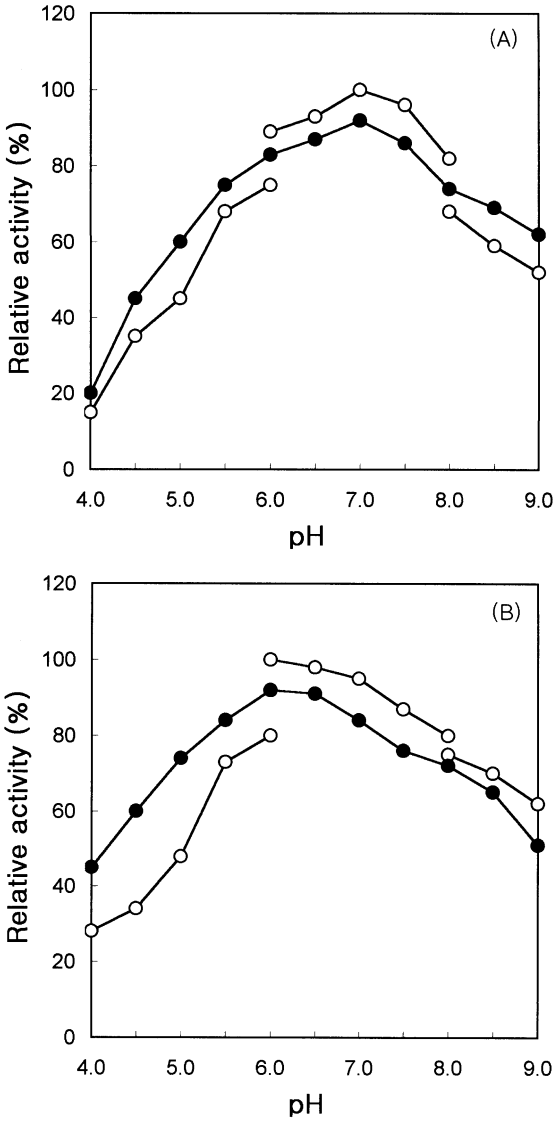

Fig. 5. Effect of pH on the hydrolysis of synthetic and natural substrates by α-galactosidase. The purified α-galactosidase was assayed using the following two series of buffers (1 ml) supplemented with 0.5% glycerol. (1) A three-buffer mixture (25 mM acetic acid, 25 mM Mes, and 50 mM Tris, -●-) titrated to the indicated pH values with KOH or HCl. (2) Acetate buffer 50 mM, pH 4.0–6.0; K-phosphate 50 mM, pH 6.0–8.0; Tris–HCl 50 mM, pH 8.0–9.0 (-○-). The pH effect was examined using PNP-α-D-Gal (A) and stachyose (B) as substrates. The relative activities calculated by two replicate assays were compared with the maximum activities (100%) that were equivalent to 39 and 14 units/mg·protein for (A) and (B), respectively.

3. Experimental

3.1. Plant materials

Grapevines of 4–5 year old *Vitis* cultivars (Muscat of Alexandria, Agawan, Marguerite, Edelweiss, Egiodola, Green hungarian, Honey black, and Takasumi) were cultivated in a greenhouse under ca. 14 h of daylight and 10 h of darkness at temperatures ranging from 18 to 31°C. Healthy, unblemished grapes were harvested up

to 3 months at intervals of 2 weeks after fruit bearing. After surface sterilization with 60% ethanol, fruits were rinsed with sterile water and immediately stored at −70°C until needed.

In order to test variations in α-galactosidase activities during postharvest storage, several bunches of grapes (*Vitis labruscana* Honey black) harvested after 9 or 12 weeks after fruit bearing were stored for 5 weeks at 4°C. Infection was minimized by repetitive rinses using 60% aq. ethanol and sterile water every 5 days during storage. At intervals of 5 days, the grapes were randomly picked from the bunches and immediately stored at −70°C until a simultaneous assay of α-galactosidase activity in flesh could be carried out. The grapes stored at −70°C just after harvest were used as controls for measuring the initial activity of the flesh.

3.2. Measurement of glycosidases

Grape flesh, separated from whole grapes, was mixed with 2% glycerol, and then directly homogenized for 30 s. The resulting paste was filtered through two layers of nylon cloth, and the filtrate was centrifuged at 8000 *g* for 20 min. After eliminating pellets, the supernatant was used for measurement of glycosidase activity in flesh. The standard assay of glycosidase was performed using PNP-glycosides as substrate. Enzyme samples were added to K-phosphate (50 mM, pH 7.0, in 1 ml of final volume) containing 0.5% glycerol. Reactions were initiated by adding 2 mM (final concentration) of substrate. After 30 min of incubation at 37°C, release of *p*-nitrophenol from the hydrolysis of substrate was detected spectrophotometrically by measuring the increase in absorbance at 410 nm (molar extinction coefficient = 1.84×10^4 M^{-1} cm^{-1}). Eventually, hydrolysis of di-, oligo-, and polysaccharides by α-galactosidase was examined by measurement of the galactose content using galactose dehydrogenase according to the method of Kurz and Kurt (1974). Enzyme sample was added to 400 μl of K-phosphate (50 mM, pH 6.0) containing 0.5% glycerol and 2 mM substrates. After incubation for 60 min at 37°C, the reaction was stopped by boiling for 5 min. The hydrolysed sample was cooled and put together with 570 μl Tris/HCl (0.1 M, pH 8.6), 20 μl of 15 mM NAD, and 10 μl galactose dehydrogenase solution (Sigma. from *Pseudomonas fluorescens*, 8.5 units/ml), with the incubation then further continued for 60 min at 37°C. The difference in Abs_{340nm} was measured from zero to the final incubation time. Quantitative analyses were performed from a calibration curve of galactose from 0 to 100 nmol. One unit of enzyme activity, in the method using PNP-glycosides, was expressed as the release of one μmol of free *p*-nitrophenol/min and specific activity as the amount released/min/mg·protein. Activity units from the method using galactose dehydrogenase were defined in the same way, except galactose was released in place of *p*-nitrophenol. Separate blanks were used for each enzyme and substrate preparation.

The pH effect of α-galactosidase was examined with 2 mM PNP-α-D-Gal or stachyose using the following buffers of similar ionic strengths: a three-buffer mixture (25 mM acetic acid, 25 mM Mes, and 50 mM Tris; Ellis and Morrisson, 1982) titrated to different pH values with KOH or HCl. Another buffer series was also used for this pH experiment: acetate buffer 50 mM, pH 4.0–6.0; K-phosphate 50 mM, pH 6.0–8.0; Tris/HCl 50 mM, pH 8.0–9.0. All buffers contained 0.5% glycerol. Hydrolysis of PNP-α-D-Gal in these buffers was determined by the standard assay method as described. The enzyme assay with stachyose was carried out in 500 μl of the two series buffers. After incubation for 1 h, each reaction mixture (200 μl) was taken to minimize the pH effect of these buffers on the subsequent galactose dehydrogenase. These samples were each added to 800 μl of the reaction mixtures for the galactose dehydrogenase assay. Further incubation and determination of galactose were performed as described. Non-enzymatic hydrolysis of PNP-α-D-Gal occurred slightly at alkaline pH, and these values were subtracted.

3.3. Extraction and purification of α-galactosidase

All purification procedures were carried out at 4°C. Grape flesh (2.6 kg) from *Vitis labruscana* Honey black was homogenized for 30 s in the presence of 0.2 M Na_2CO_3, 1% (w/v) insoluble polyvinylpolypyrrolidone, and 5% glycerol. The homogenate was filtered through two layers of nylon cloth. The resulting filtrate was centrifuged at 8000 *g* for 20 min and the pellet discarded. The supernatant was fractionated with ammonium sulfate at 30–55% saturation. The precipitate was recovered by centrifugation at 10,000 *g* for 20 min and resuspended in 100 ml of 40 mM potassium phosphate buffer (pH 7.0) containing 2% glycerol (buffer A). Clarification was done by dialysis with the same buffer and insoluble materials were removed by centrifugation at 10,000 *g* for 20 min.

The clarified supernatant was applied to a Biogel P-60 (Bio-Rad) column equilibrated with buffer A. Proteins were eluted with the same buffer at a flow rate of 12 ml/h. An additional aim of this step prior to affinity chromatography was to minimize various small substances such as pigments, which may interfere with chromatographic behavior by nonspecific adsorption. α-Galactosidase activity was monitored using PNP-α-D-Gal as a substrate throughout the purification procedures including this step.

The active fractions obtained were loaded onto a melibiose-agarose (Sigma) column (2 × 10 cm) equilibrated with buffer A. After washing the column, proteins were eluted with 200 ml of buffer A containing a linear gradient of K-phosphate (0–500 mM) at a flow rate of 15

ml/h. Fractions with α-galactosidase activity were collected and clarified by centrifugation for next step.

The pooled fractions from the affinity chromatography were further purified using Sephacryl S-200 (Pharmacia) chromatography. Proteins were eluted with 50 mM K-phosphate buffer (pH 7.0) containing 2% glycerol at a flow rate of 12 ml/h. Purification was followed by monitoring the absorbance at 280 nm and assaying enzyme activity. Except for physiological aspects, all of the experiments on α-galactosidase were performed using this sample obtained from this chromatography.

3.4. Native molecular mass determination

The apparent molecular mass of the purified α-galactosidase was estimated by gel filtration chromatography using a Sephacryl S-200 column (2 × 90 cm), which was equilibrated with 50 mM K-phosphate buffer (pH 7.0) containing 2% glycerol. The following proteins were co-injected with the enzyme to the column for the calibration: aldolase (M_r; 158,000), phosphorylase b (97,400), BSA (66,200), ovalbumin (43,000), and cytochrome c (12,000). The proteins were eluted with the same buffer at a flow rate of 12 ml/h. Along with measuring Abs_{280nm}, the activity peak was monitored by assaying the enzyme activity using PNP-α-D-Gal as substrate.

3.5. Other analytical methods

Soluble solid contents were measured with a refractometer using 50 μl of flesh juice, that was prepared by slightly grinding and subsequent centrifugation at 10,000 *g* for 5 min. Reducing sugars produced from the hydrolysis of polygalacturonic acid were measured using 2-cyanoacetamide (Gross, 1982). SDS-PAGE was performed according to the method of Laemmli (1970). A slab gel that consisted of 12.5% acrylamide resolving gel and 5% acrylamide stacking gel was used, with the gel stained with Coomassie Brilliant Blue R-250 and then with silver nitrate (Wray et al., 1981). Protein concentrations were determined by the dye-binding assay of Bradford (1976) using BSA as a standard protein.

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