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Influence of Gibberellic Acid on α-D-Galactosidase Activity in the Grape Berry

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Glycosidase activities in the grape flesh (Marguerite) were assayed, and the order of activity was marked as follows: α-D-galactosidase>α-D-mannosidase>α-D-glucosidase>β-D-galactosidase>β-D-glucosidase. Of these glycosidases, α- and β-D-galactosidases were prominently expressed by the treatment of gibberellic acid, resulting in 56 and 238% increase of activity, respectively. Most of α-D-galactosidase was found in the flesh texture, and the activity increase by gibberellic acid occurred mostly in this tissue. The increase in α-D-galactosidase activity was dependent on the concentration of gibberellic acid treated, showing a positive correlation. Gibberellic acid affected the content of total protein in the grape flesh, 49% increase by 75 ppm treatment. Above this concentration, higher gibberellic acid level did not influence the protein expression. Specific activity of the α-D-galactosidase still increased, showing 24% increase in activity. Grape flesh subjected by gibberellic acid (100 ppm) resulted in the increased activity against a natural substrate, stachyose, showing 55% increase in activity from the grapes treated with 100 ppm of gibberellic acid. Other natural substrates, such as melibiose and raffinose, were also considerably hydrolyzed, and the extent was similar to that of stachyose hydrolysis. During postharvest storage, α-D-galactosidase activity in the grape flesh increased by 51% after 20 days and then declined slowly.

Key words: *gibberellic acid, α-D-galactosidase.*

Gibberellins are a class of endogenous phytohormones that regulate a wide range of developmental processes in plants, including germination, stem elongation, and flower development. Most molecular studies on the mechanism of auxin and gibberellic acid action have been focused on the induction of cell elongation in vegetative tissues such as stems, coleoptiles, hypocotyls, and epicotyls.[1,2] Some of these phytohormone-induced genes have been characterized, and the functions of proteins have been investigated.[3,4] In phytohormone-induced gene expression, the most widely investigated protein includes the induction of α-amylase by gibberellins in cereal germinants.[5] Other researches have shown that gibberellins also influence the biosynthesis of various hydrolytic enzymes involved in protein and lipid degradations.[5-10]

Gibberellic acid has been routinely used in seedless bunch grape production[11] and can be used to increase berry and bunch sizes.[12,13] Treatment to some fruit with gibberellic acid also can delay ripening.[14,15] β-Galactosidase that may facilitate the cell growth was shown to be increased by auxin in *Avena*[16] and etiolated pea stems.[17] Galactose elicits various physiological responses related with ethylene and auxin from mung bean hypocotyls,[18] *Avena* coleoptiles,[19] and tobacco leaf discs.[20] On the analogy of these findings, treatment of gibberellin as one of phytohormones could cause some variations of glycosidases in the grape berry in relation with the plant growth regulation.

Despite wide-spread investigation on the role or mechanism of gibberellic acid in plants, induction of glycosidase by the plant growth regulator has been relatively less focused on fruits. Recently, the expression of invertase by gibberellic acid was reported in relation with the berry-sizing effect,[21] berry development, and cell compartmentation.[22] In order to understand the role of gibberellic acid, fruit ripening, and mechanism of proteins expressed, it is necessary to assess glycosidases induced by the phytohormone. Thus, we report here some characteristics of glycosidase, that was expressed in the grape flesh by the application of gibberellic acid.

Materials and Methods

Plant materials. Five-year-old *Vitis* cultivars (Takasumi, Marguerite, Agawan, and Edelweiss) were cultivated in a green house under 14 h of day light and 10 h of darkness at temperatures ranging from 21 to 30°C. Fully ripe grapes were harvested after about 3 months of growth from berry setting, and healthy and unblemished grapes were selected. After surface sterilization with 60% ethanol, fruits were rinsed with sterile water, and immediately stored at -70°C until use.

Treatment of gibberellic acid. In order to test the influ-

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Abbreviations: PNP, p-nitrophenyl.

ence of gibberellic acid on the glycosidase expression, grape blossoms were dipped into the gibberellic acid solution (0-100 ppm) 10 days before and 15 days after blooming. The grapes reaching full-ripe stage (12 weeks after the berry setting) were harvested concurrently, regardless of ripening states caused by the different concentration of gibberellic acid subjected.

Postharvest storage. In an attempt to test the variation of α-D-galactosidase activity during postharvest storage, several bunches of grapes (Takasumi) harvested after 3 months from the berry setting were stored for 5 weeks at 4°C. The fruits were disinfected as much as possible by repetitively rinse with 60% ethanol and sterile water every 5 days during storage. The grape berries were randomly picked from the bunches every 5 days during postharvest storage, and immediately stored at -70°C until simultaneous assay of α-D-galactosidase activities of the flesh. Grapes stored at -70°C just after harvest were used as control grapes for measuring the initial activity of the flesh.

Preparation of glycosidase assay sample. For the preparation of glycosidase assay sample, exocarp and flesh were separately isolated from the grapes. The exocarp was homogenized for 2 min in the presence of one volume (w/w) 50 mM K-phosphate (pH 7.0), 0.2 M Na_2CO_3, and 0.5% glycerol. The resulting paste was filtered through two layers of nylon cloth, and the filtrate was centrifuged at 8,000 g for 20 min. After eliminating the pellets, the supernatant was used for the measurement of glycosidase activities in the exocarp. Crude extract from the flesh was prepared by the same method, except for direct homogenization without any additive buffer. For the test of glycosidase distribution, the flesh fraction was further separated into flesh juice and texture fractions. For this separation, the flesh was cut into five to seven pieces per berry, and the flesh juice was filtrated with nylon cloth. Supplementary juice was obtained from the resulting paste by centrifugation at 8,000 g for 20 min and mixed with the juice earlier and 0.5% glycerol. The resulting mixture was used as a fraction of flesh juice. The resulting pellet remained formed during the flesh juice preparation was homogenized for 2 min after addition of one volume of 50 mM K-phosphate (pH 7.0), 0.2 M Na_2CO_3, and 0.5% glycerol. The resulting homogenate was filtered with nylon cloth and centrifuged at 8,000 g for 20 min. The supernatant thus obtained was used as a flesh texture fraction.

Measurement of glycosidase activity. Activity of the glycosidases was routinely assayed using *p*-nitrophenyl glycosides (PNP-glycosides) as the substrate. Test samples were added to the reaction mixture (1 m*l* of final volume) consisting of 50 mM K-phosphate (pH 7.0), 0.5% glycerol, and 2 mM substrate. After 30 min of incubation at 37°C, the release of *p*-nitrophenol was detected spectrophotometrically by measuring the increase in absorbance at 410 nm (molar extinction coefficient = $1.84 \times 10^4\ M^{-1} \cdot cm^{-1}$).

As an alternative glycosidase assay, hydrolytic activity of α-D-galactosidase against di-, oilgo-, and polysaccharides was examined through the measurement of galactose content using galactose dehydrogenase according to the method of Kurz and Kurt.[23] α-D-Galactosidase sample was added to 400 μ*l* of the reaction mixture comprising 50 mM K-phosphate (pH 6.0), 0.5% glycerol, and 2 mM substrates. After incubation for 60 min at 37°C, the reaction was stopped by boiling for 5 min. Following cooling down, the hydrolysed sample was put together with the subsequent reaction mixture consisting of 570 μ*l* 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^{-1}), and the incubation further continued for 60 min at 37°C. The difference in Abs 340 nm was measured from zero to final incubation time. Quantitative analysis was performed using a calibration curve of galactose from 0 to 100 nmol.

In the both assays, each unit of the α-D-galactosidase activities from the two methods were defined as the hydrolysis of one μmol of free *p*-nitrophenol per min and galactose per min, respectively. Separate blanks were used for each enzyme and substrate preparation. All the enzyme activities were represented as mean values of three dependent experiments.

Other analytical methods. Protein concentrations were determined through the dye binding assay method of Bradford[24] using BSA as a standard protein. Soluble solids content was tested with a refractometer using 50 μl of flesh juice, that was prepared by slight grinding and centrifugation at 10,000 g for 5 min.

Results and Discussion

Glycosidase activities in the grape flesh. Various glycosidase activities were assessed from the grape flesh (Marguerite) that were treated with and without gibberellic acid (Table 1). Among the glycosidases in the grape flesh naturally developed, α-D-galactosidase activity was the most active, followed by α-D-mannosidase, α-D-glucosidase, β-D-glucosidase, and β-D-galactosidase. But treatment of gibberellic acid, on the whole, resulted in the increased activities of glycosidase. At 100 ppm of gibberellic acid, α-D-galactosidase showed still the highest activity, and other glycosidases expressed by the following order: β-D-galactosidase>α-D-mannosidase>α-D-glucosidase>β-D-glucosidase.

To obtain a more generalized feature on the glycosidase expression, the enzyme activities in the flesh were compared between three grape strains treated with or without 100 ppm gibberellic acid (Table 2). In all strains tested, the highest activity of α-D-galactosidase was common, regardless of the treatment of gibberellic acid, and α-D-galactosidase was also significantly induced by the phytohormone.

Distribution of α-D-galactosidase. α-D-Galactosidase activity was assayed from the fractions isolated into flesh texture, flesh juice, and exocarp, that were prepared from the grape (Marguerite) (Table 3). In all the fractions, the activity in the α-D-galactosidase increased by the treatment of gibberelic acid (100 ppm). Expression of α-D-galactosidase was the most prominent in the flesh texture fraction, showing 60%

Table 1. Glycosidase activities in the grape flesh (Marguerite), treated with and without gibberellic acid. The enzyme assay was run by hydrolysis of PNP-glycosides (2 mM) in 50 mM K-phosphate (pH 7.0) and 0.5% glycerol. Glycosidase activities are represented as units per g · fresh wt. Soluble solids content of the grape berry was 17.1°Bx.

Substrate	Glycosidase activity (unit/g flesh wt)	
	Non treatment	Gibberellic acid (100 ppm)
PNP-α-D-Gal	149.1	232.6
PNP-β-D-Gal	45.0	152.3
PNP-α-D-Glc	64.5	76.1
PNP-β-D-Glc	51.9	62.3
PNP-α-L-Fuc	32.7	28.7
PNP-β-D-Fuc	18.5	19.6
PNP-α-D-Man	87.4	102.6
PNP-α-L-Ara	6.7	5.4
PNP-α-L-Rha	<1.0	<1.0
PNP-β-D-GlcUA	<1.0	<1.0
PNP-α-D-GlcNAc	<1.0	<1.0
PNP-β-D-GlcNAc	<1.0	<1.0
PNP-β-D-GalNAc	22.4	21.9
Polygalacturonic acid	2.8	3.1

Note: Polygalacturonase activity was assayed using 2-cyanoacetamide.[26]

increase in the enzyme activity. But the extent of increase was not considerable in the flesh juice or exocarp. With regard to protein extraction from the grape flesh, we would like to note that homogenization with 0.2 M Na_2CO_3 resulted in the increase of α-D-galactosidase activity by 2.9 times. Alternatively, addition of 0.05 N NaOH caused the enzyme activity to increase by as much as 2.5 times (detailed data not shown). These results indicate that the increas in ionic strength and/or the augmentation of pH release more α-D-galactosidase from the grape flesh texture. These results suggest that α-D-galactosidase among the isoenzymes in the exo- and endocarps might be associated mainly with the grape texture, and the enzyme induction by gibberellic acid occurs prominently in this texture tissue.

Table 3. Distribution of glycosidases in the grape berry, treated without and with gibberellic acid. The grape (Marguerite) was treated with gibberellic acid 10 days before and 15 days after blooming. α-Galactosidase activity was represented as unit/g fresh wt. Soluble solids content of the grape berry was 18.2°Bx. The ratio of texture to juice was 91 : 9 (w : w) in the grape flesh.

Gibberellic acid (ppm)	α-Galactosidase activity (unit/g fresh wt)		
	Flesh texture	Flesh juice	Exocarp
None	701.1	44.5	45.7
25	829.2	56.1	46.4
50	994.8	62.5	50.1
100	1124.6	68.4	49.8

Expression of α-D-galactosidase during the development stage. Figure 1 shows the variation in the α-D-galactosidase activity per g flesh from the grape flesh (Takasumi) harvested at different stages of ripening. Regardless of gibberellic acid treatment, the α-D-galactosidase activities were scarcely found in the unripe grape flesh (approximately up to 4 weeks from the fruit bearing). After this stage, the α-D-galactosidase activity increased rapidly in the grape fleshes of 100 ppm gibberellic acid. After 12 weeks, α-D-galactosidase activity increased by 12-fold. In the grapes developed naturally without gibberellic acid, the α-D-galactosidase activity also steadily increased along with the development, but the extent of increase was less compared with the grape treated with gibberellic acid, showing 7-fold increase during the same period of development.

The highest expression of α-D-galactosidase synchronizing with the ripening stage could be correlated with the report of Gross,[25] which revealed that exogenously applied galactose stimulated the ethylene production and promoted the ripening of mature green tomatoes. Similarly to our result, an invertase activity increased by the application of gibberellic acid and stimulation of the enzyme was suggested to be involved in the

Table 2. Glycosidase activities in the flesh from diverse grape cultivars, treated with and without gibberellic acid. Glycosidase assay was conducted using PNP-glycosides (α- and β-D-gal, α- and β-D-Glc, α-L-and β-D-Fuc, α-D-Man, α-L-Ara, α-L-Rha, α-D-GlcUA, α- and β-D-GlcNAc, and β-D- GalNAc) and polygalacturonic acid as substrates. Five substrates, that were hydrolyzed rapidly are shown by the order of activity (unit/mg protein).

Strains		Orders of glycosidase activity (unit/mg protein)
Marguerite	(none)	α-D-Gal(149) ··· α-D-Man(101) ··· α-D-Glc(75) β-D-Gal(62) ··· β-D-Glc(51)
	(100 ppm)	α-D-Gal(225) ··· β-D-Gal(190) ··· α-D-Man(184) β-D-Glc(98) ··· β-D-Glc(72)
Agawan	(none)	α-D-Gal(453) ··· α-D-Man(321) ··· α-D-Glc(298) β-D-Gal(221) ··· β-D-Glc(176)
	(100 ppm)	α-D-Gal(564) ··· β-D-Gal(379) ··· α-D-Man(321) α-D-Glc(290) ··· β-D-Glc(246)
Edelweiss	(none)	α-D-Gal(202) ··· α-D-Man(175) ··· β-D-Gal(121) α-D-Glc(99) ··· β-D-Glc(72)
	(100 ppm)	α-D-Gal(379) ··· β-D-Gal(203) ··· α-D-Man(183) α-D-Glc(127) ··· β-D-Glc(102)

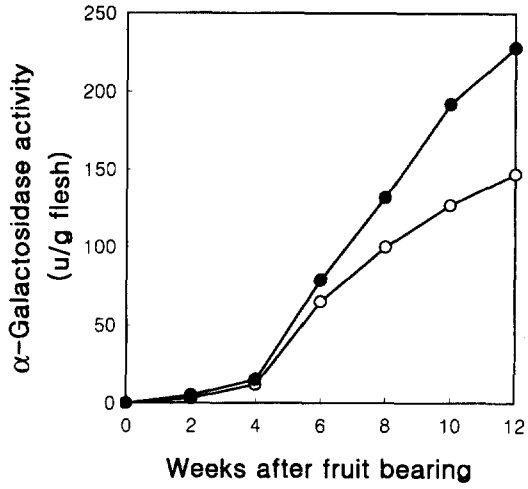

Fig. 1. α-D-galactosidase activity of the grape flesh (Takasumi) at different stages of development. The grape blossoms were soaked without (- ○ -) and with 100 ppm of gibberellic acid (- ● -) 10 days before and 15 days after the blooming. After 12 weeks from the berry setting, the grapes were harvested, and α-D-galactosidase activity was assayed.

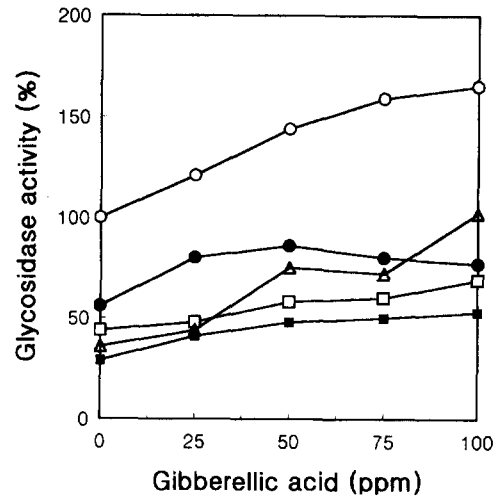

Fig. 2. Influence of gibberellic acid concentration on various glycosidase activity in the grape (Takasumi) flesh. The grape blossoms were soaked in different concentrations of gibberellic acid (from 0 to 100 ppm) 10 days before and 15 days after blooming. After 12 weeks from the berry setting, the grapes were harvested, and the flesh fractions were prepared for enzyme assay. α-D-Galactosidase (- ○ -), β-D-galactosidase (- △ -), α-D-mannosidase (- ● -), α-D-glucosidase (- □ -), and β-D-glucosidase (-■ -) activities are presented as relative activities. α-D-Galactosidase activity of the non-treated grape was considered as 100%, corresponding to 152 unit per g tissue.

berry-sizing effect.[21]

Influence of gibberellic acid concentration on the α-D-galactosidase expression. Expression of diverse glycosidase was examined from the grapes subjected with different concentrations of gibberellic acid (Fig. 2). In general, gibberellic acid stimulated the expression of all glycosidases tested in a concentration-dependent manner. Among these enzymes, the α-D-galactosidase was still the most active and showed 64% increase after the 100 ppm treatment. Other glycosidases, β-D-galactosidase, α-D-mannosidase, α-D-glucosidase, and β-D-glucosidase, were also considerably expressed by the gibberellic acid treatment. But none reached to the expressed level of α-D-galactosidase. The expression rate of β-D-galactosidase, however, was the most prominent, showing 2.7 times increase in activity by the 100-ppm treatment.

In order to further characterize the α-D-galactosidase expression by gibberellic acid, biosynthesis of total protein per mg flesh was tested, and specific activity of α-D-galactosidase was plotted (Fg. 3). Gibberellic acid caused more production of total protein in the grape flesh, to the extent of 49% increase by 75-ppm treatment. Above this concentration level, gibberellic acid did not augment the protein synthesis. The profiles of proteins expressed by gibberellic acid was not searched in this experiment. Specific activity (unit/mg protein) in the α-D-galactosidase was calculated to be still augmented, showing 24% increase in activity to a lesser extent than the total activity (unit/g flesh).

The expression patterns of glycosidases in the flesh differ, based on the total and specific activities, from each other between the grapes, which were cultivated with and without gibberellic acid. In the grape treated without gibberellic acid, the glycosidases increased in the activities per g flesh as well as specific activities (unit/mg protein) along with ripening

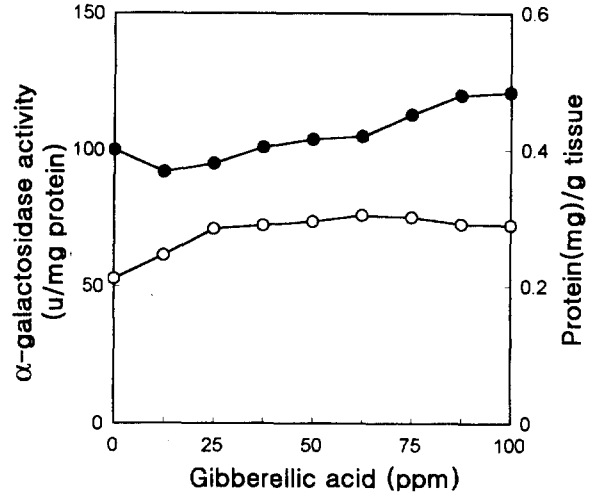

Fig. 3. Variation of specific α-D-galactosidase activity in the grape flesh (Takasumi) treated with gibberellic acid. The grape blossoms were soaked in different concentrations of gibberellic acid (from 0 to 100 ppm) 10 days before and 15 days after blooming. After 12 weeks from the berry setting, the grapes were harvested, and the flesh fractions were prepared for enzyme assay. Variation of protein concentration (- ○ -) and α-D-galactosidase activity/mg protein (- ● -) were measured as described in the Materials and Methods.

stage. But in the gibberellic acid treated grapes, the specific activities only slightly increased by gibberellic acid, despite the marked augmentation of activities per g flesh. This difference might be explained by the reason that more wide range of proteins, in addition to glycosidases, might be expressed since

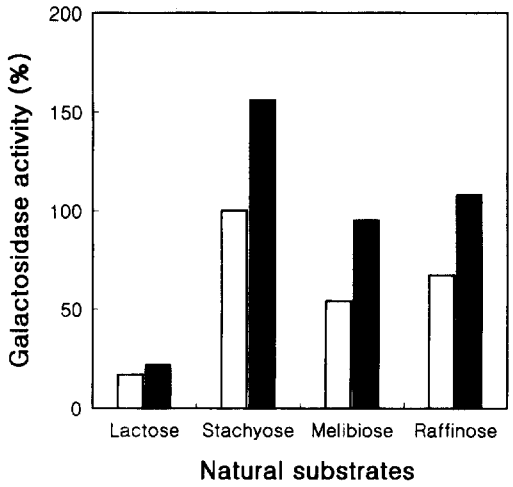

Fig. 4. Hydrolysis of natural substrates by the grape (Takasumi) flesh treated with and without gibberellic acid. Assay sample was incubated with galactose related carbohydrates (2 mM). The subsequent reaction and estimation of galactose released were carried out through the galactose dehydrogenase assay as described in Materials and methods. The activities were compared with the hydrolysis of stachyose, which was 21 unit/mg protein. Empty and filled bars represent the grapes which were treated withiout and with 100 ppm of gibberellic acid, respectively. All values were the means of three determinations.

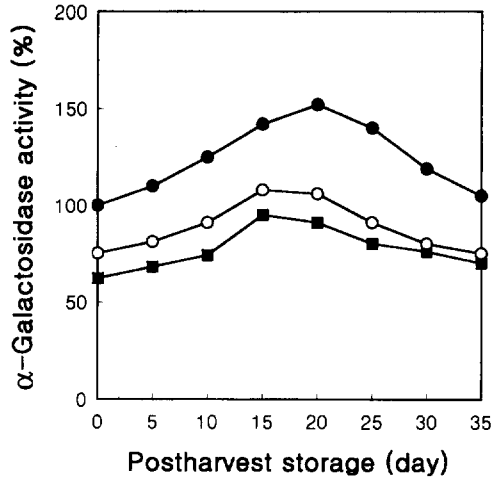

Fig. 5. Activity variation of α-D-galactosidase in the grape flesh during postharvest storage. The grapes (Takasumi) applied with 0 (-■-), 50 (-○-), and 100 ppm (-●-) of gibberellic acid were harvested after 12 weeks from berry setting and stored at 4°C. The activity of α-D-galactosidase was assayed at the same time. One hundred percent activity was 109 unit per g tissue. Protein concentration was roughly invariable during the postharvest storage. Soluble solids contents increased in proportion with the storage period, showing from 12.4 to 14.9 °Bx.

total protein per g flesh increased in the flesh by the addition of gibberellic acid.

Soluble solids contents in the grape flesh (Takasumi) steadily decreased by the application of gibberellic acid, from 15.4 to 13.8°Bx by 0 to 100 ppm treatment, respectively (detailed data not shown). Weight of berry, however, increased proportionally to the increase in gibberellic acid concentration, showing from 2.4 to 3.9 g per berry by the treatment of 0 to 100 ppm (detailed data not shown). Collectively, application of gibberellic acid induced the protein expression including the glycosidases and decreased the soluble solids contents along with the increase in gibberellic acid subjected. In general, glycosidases might be involved, at least partly, in the ripening of fruits including grape berry. The glycosidase in the grape might not play an exclusive role in the ripening process since the increase in glycosidase activities in the grape flesh has a negative correlation with the soluble solids content which is considered as a prime index in the determination of ripening state. On the contrary, the increase in glycosidase activities showed a positive correlation with the grape berry size. The results reported here provide a new insight into the role of gibberellic acid in stimulating the grape berry size rather than in the ripening process.

Hydrolysis of natural substrates. Glycosyl-PNP, that is widely used for glycosidase assay, is a synthetic substrate. The oligo~polysaccharides existing in the grape flesh might be considerably hydrolyzed by glycosidases including α-D-galactosidase during the developmental stage of grape. Thus, isolation of these natutral substrate and subsequent hydrolysis by glycosidase might be less meaningful. Therefore, the expression of α-D-galactosidase was examined using natural substrates including stachyose (Fig. 4). In the grape cultivated at 100 ppm gibberellic acid, the galactosidase activity was augmented by 55% against stachyose. Hydrolysis rates of melibiose or raffinose were also considerably augmented, and the extent of increase was similar to that of stachyose hydrolysis.

Variation of α-D-galactosidase during postharvest storage. Putative variation of α-D-galactosidase activity during postharvest storage was examined using the grapes (Takasumi), that were treated with gibberellic acid (Fig. 5). At 100 ppm treatment, the α-D-galactosidase activity increased by 51% in the grape flesh after 20 days of storage, and then declined slowly. The enzyme activity also increased similarly in the grapes cultivated after subjection of 0 or 50 ppm. During the storage at room temperature, the activity increased more rapidly than at 4°C, and then decline. But difficulty in storage at room temperature hampered the test for the programmed period (data not shown).

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