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Stability to Light, Heat, and Hydrogen Peroxide at Different pH Values and DPPH Radical Scavenging Activity of Acylated Anthocyanins from Red Radish Extract

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The stability of red radish extract to light, heat, and hydrogen peroxide at different pH values (3, 5, and 7) was examined, in which major anthocyanins were pelargonidin glycosides acylated with a combination of *p*-coumaric, ferulic, or caffeic acids. The light irradiation (fluorescence light, 5000 lx; at 25 °C) indicated that the red radish extract was more stable at lower pH than at higher pH. The HPLC analyses revealed that diacylated anthocyanins in the extract were more stable to light at pH 3 than monoacylated anthocyanins. No significant difference in degradation rates of acylated anthocyanins at pH 5 was observed, whereas anthocyanins acylated with *p*-coumaric or ferulic acids were more stable at pH 7 than ones with caffeic acids. The stability to heat (at 90−95 °C) showed a tendency similar to that for light. The number of intramolecular acyl units contributes to stability to light and heat at lower pH, whereas the characteristics of intramolecular acyl units influence the stability at higher pH. The degradation behavior of red radish extract to H_2O_2 were almost the same to those of light and heat, depending on the pH. However, HPLC analyses revealed that the stability of individual acylated anthocyanins were independent of the pH. These data suggest that the characteristics, the number, and the binding site of intramolecular acyl units affect the stability of anthocyanin to H_2O_2. DPPH radical scavenging activity of all acylated anthocyanins was higher than those of pelargonidin and perlargonidin-3-glucoside. The activity of acylated anthocyanins mostly depended on the activity of intramolecular acyl units (caffeic acid > ferulic acid > *p*-coumaric acid). However, the activity was highly affected by the binding site of intramolecular acyl units even if anthocyanins have common acyl units.

KEYWORDS: Acylated anthocyanin; acylated pelargonidin; stability; antioxidant activity; red radish

INTRODUCTION

Anthocyanins are water-soluble plant extracts responsible for red, purple, and blue colors and mainly distributed among flowers, fruits, and vegetables. Recently, there has been increasing use of anthocyanin pigments instead of synthetic dyes, because of the increasing awareness of consumers regarding food safety (*1*, *2*). In addition, they have beneficial biological properties such as enhancement of sight acuteness (*3*), antioxidative (*4*−*6*), and anticarcinogenic effects (*7*, *8*). The properties are expected to protect living organisms from several types of damage, resulting in the prevention of diseases. However, some limitations restrict the use of anthocyanins because of low stability to several processing and storage conditions.

The anthocyanidins (aglycones) are characterized as having the flavinium (2-phenylbenzopyrium) cation structure and different hydroxyl or methoxyl substitutions on the B-ring. The naturally occurring anthocyanins are glycosides and acylglycosides of anthocyanidins. There are numerous reports on the stability of anthocyanins, and several theories have been proposed: self-association, copigmentation, and intramolecular copigmentation (*9*). It is thought that acylation with aromatic acids makes the anthocyanins more stable through intramolecular stacking of the aromatic acid to the anthocyanidin nucleus by hydrophobic interactions. Hayashi et al. (*10*) examined heat and UV irradiation stabilities of anthocyanins using 19 kinds of vegetables and fruits and concluded that the anthocyanin composition and the proportion of acylation affected the stability of anthocyanins. Redus et al. (*11*) reported that the side-chain double bond and substituents of cinnamic acids affected the stability of monoacylated anthocyanins. Giusti and Wrolstad (*12*)

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discussed the relationship between chemical structure of some acylated anthocyanins and the stability and reviewed their potential applications in the food system.

Acylated anthocyanins also have higher antioxidant ability than the corresponding anthocyanidins and their glycosides (*13*−*16*). In addition, recent studies support that some acylated anthocyanins can be absorbed by rats and humans as intact acylated forms (*17*−*21*). Wu et al. (*22*) have reported that acylated anthocyanins contributed 23% of total daily anthocyanin intake according to concentrations in common foods in the U.S. There is, however, little information regarding the relationship between chemical structure of acylated anthocyanins and the stability or antioxidant activity because acylated anthocyanins are compounds with complex patterns of glycosylation and acylation and are not commercially available.

The red radish (*Raphanus sativus* L.) is widely used as a vegetable and for natural food colors. The red radish extracts impart orange-red colors, similar to FD&C No. 40, a synthetic food colorant. It contains a significant amount of anthocyanins, the major components being acylated pelargonidin glycosides. Giusti et al. (*23*) have reported that four acylated pelargonidins were identified from aqueous acetone extracts of the red radish. Wu and Prior (*24*) have reported that a total of 34 anthocyanins were detected from the extraction with a mixed solution of methanol:water:acetic acid. In our previous report, 12 anthocyanins acylated with a combination of *p*-coumaric, ferulic, or caffeic acids were identified from 0.05% H_2SO_4 extracts (*25*). Some reports also support our results (*22*, *26*).

In this study, we examined stabilities of the individual anthocyanins in red radish extract to light, heat, and hydrogen peroxide at different pH values. In addition, the antioxidant activities of acylated pelargonidins were investigated. We discuss the effects of acyl units on the stability and antioxidant activity of anthocyanins.

MATERIALS AND METHODS

Materials. Red radish extract (Mitsubishikagaku Foods Non Commercial Product) was obtained from Mitsubishikagaku Foods Corporation (Kanagawa, Japan). Hydrogen peroxide and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Pelargonidin chloride and pelargonidin-3-*O*-glucoside chloride (callistephin chloride) were from Extrasynthese Co. (Genay Cedex, France). The acylated anthocyanins were isolated from red radish by column chromatography, followed preparative HPLC as reported previously (*25*). All reagents were of HPLC or special grade and purchased from Wako Pure Chemical Ind.

Analysis of Anthocyanins in Red Radish Extracts. The anthocyanins in red radish extract were analyzed by HPLC as previously reported (*25*). The column was a Capcell Pak C18 column (4.6 × 250 mm, 5 μm, Shiseido Fine Chemicals, Tokyo, Japan), and the mobile phase consisted of solvents A (1.5% H_3PO_4) and B (1.5% H_3PO_4, 20% CH_3COOH, 25% CH_3CN), starting with 80 min isocratic at 30% solvent B, followed by a gradient to 45% solvent B at 120 min, and isocratic 45% solvent B for 70 min at flow rate of 1.0 mL/min. The column temperature was maintained at 40 °C, and the effluent was monitored at 300−600 nm using a Waters 996 photodiode array detector (Tokyo, Japan). When quantitative determination of individual anthocyanins was done, the effluent was monitored at 520 nm. During storage, residual anthocyanin contents were estimated from absorbance at $\lambda_{vis-max}$ and degradation index (DI value) to determine colorant degradation. The absorption spectra were scanned from 350 to 700 nm with a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). The DI value was calculated as the ratio between absorbance at 420 nm and at $\lambda_{vis-max}$ (*27*). The individual acylated anthocyanins were analyzed by HPLC described above, and the residual anthocyanins (%) were calculated as the percent of initial peak area.

Photostability Test. The red radish extract was dissolved in McIlvaine buffer at different pH values (3, 5, and 7). Samples (0.2 w/v%) were placed into 15 mL glass vials with screw caps and exposed to light (fluorescent light, 5000 lx; a photo period of 24 h/d) in an incubator (Hitachi CT-30) at 25 °C for different time periods. Residual anthocyanin contents were determined using a UV−vis spectrophotometer and HPLC. Throughout the storage period, the samples were randomly interchanged once every day to minimize the effects of unequal exposure.

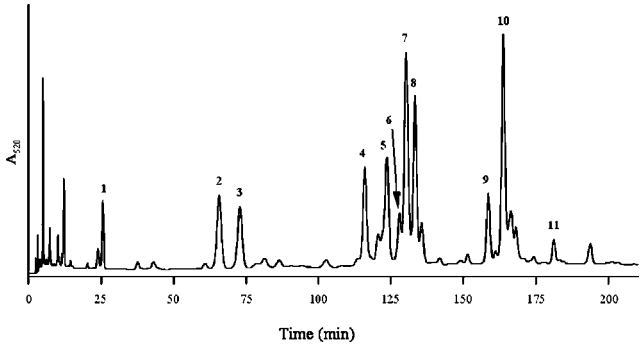


Figure 1. HPLC profile of red radish extract.

Thermostability Test. The red radish extract was dissolved in McIlvaine buffer (pH 3, 5, and 7). Samples (0.2 w/v%) were placed into a glass tube with screw cap, wrapped in tin foil, and then immersed in a water bath at 90−95 °C for different time periods. After each sample was cooled in an ice−water bath, residual anthocyanin contents were determined by using a UV−vis spectrophotometer and HPLC.

Stability Test to Hydrogen Peroxide. Hydrogen peroxide (30, 60, and 300 μg/mL) was added to red radish extract (0.2 w/v%) dissolved in McIlvaine buffer (pH 3, 5, and 7). Samples were stored at 25 °C in the dark for different time periods. Residual anthocyanin contents were determined using UV−vis spectrophotometer and HPLC.

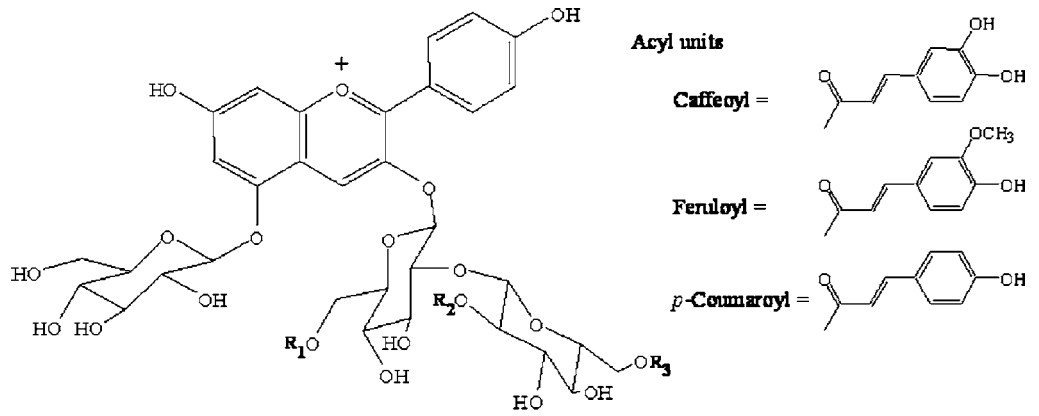
DPPH Radical Scavenging Activity. DPPH radical scavenging activity of nine isolated acylated anthocyanins was measured according to the method of Yamaguchi et al. (*28*) with some modifications. Each acylated anthocyanin was dissolved in 0.1% TFA−MeOH solution to 40 μM concentration. An aliquot (20 μL) of sample solution was added to McIlvaine buffer (pH 3 and 7; 80 μL) and 100 μmol/L of DPPH−EtOH solution (100 μL). After standing for 20 min in the dark at room temperature, the reaction sample (50 μL) was applied to HPLC system. The activity was evaluated by measuring the decrease of DPPH detected by HPLC. HPLC analysis run on a Zorbax C8 (4.6 × 250 mm, Rockland technology) column at 40 °C at a flow rate of 1 mL/min using 90% MeOH solvent as the mobile phase at 517 nm.

Experiment. All experiments were done in at least triplicate. The data presented are the means of replicate experiments. Statistical significance of the results was evaluated using ANOVA and Tukey multiple-range test at $p < 0.05$. All statistical analyses were performed in GraphPad Prism for Windows, GraphPad software.

RESULTS

Color Properties and Anthocyanins in a Red Radish Extract. Anthocyanin profile in the red radish extract is shown in **Figure 1**. Peak assignments were done by comparison of the retention time and UV−vis spectra and coinjection analyses with the compounds isolated previously (*25*). Although many peaks were observed in the chromatogram, major peaks were identified as acylated anthocyanins reported previously; pelargonidin-3-sophoroside-5-glucoside with a combination of *p*-coumaric, ferulic, or caffeic acids (**Figure 2**). The contents of the acylated anthocyanins (**1**−**11**) were estimated as 33, 98, 84, 118, 120, 50, 223, 159, 65, 220, and 20 mg/100 g, respectively, from each calibration curve.

Chromaticity of Red Radish Extract at Different pH Values. UV−vis spectra of red radish extract at different pH values are shown in **Figure 3**. Hypochromic effects were observed at pH < 5 and hyperchromic effects and bathochromic shifts were observed at pH > 5. It is well-known that



1	R_1=H	R_2=H	R_3=Feruloyl
2	R_1=Caffeoyl	R_2=H	R_3=H
3	R_1=Caffeoyl	R_2=H	R_3=Caffeoyl
4	R_1=Caffeoyl	R_2=H	R_3=Feruloyl
5	R_1=*p*-Coumaroyl	R_2=H	R_3=H
6	R_1=*p*-Coumaroyl	R_2=H	R_3=Caffeoyl
7	R_1=Feruloyl	R_2=H	R_3=H
8	R_1=Feruloyl	R_2=H	R_3=Caffeoyl
9	R_1=*p*-Coumaroyl	R_2=H	R_3=Feruloyl
10	R_1=Feruloyl	R_2=H	R_3=Feruloyl
11	R_1=Feruloyl	R_2=Feruloyl	R_3=H

Figure 2. Chemical structures of acylated anthocyanins in red radish extract.

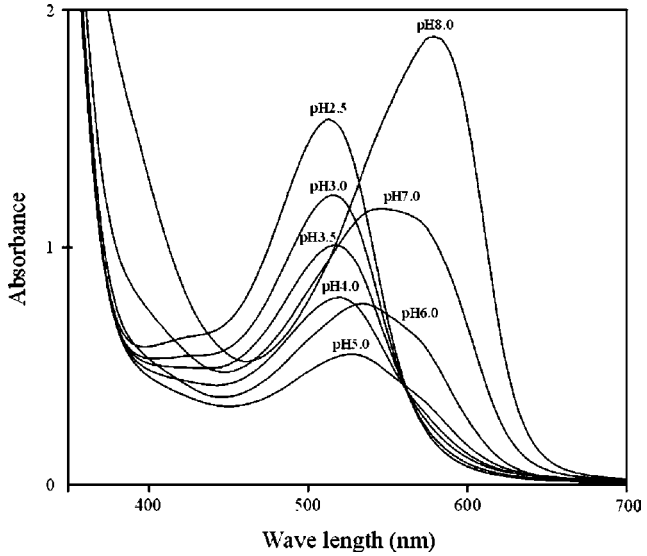


Figure 3. UV–vis spectra of red radish extract at different pH values.

anthocyanins exist in an aqueous solution as a mixture of four molecular species, depending on the pH; red flavylium cation, colorless carbinol pseudo-base, blue-purple quinoidal-base, and pale yellow chalcone. Thus, for the red radish extract, the flavylium cation was hydrated to yield the carbinol pseudo-base at pH 3−5 and the quinoidal-base at pH 5−8. On the other hand, there was no difference in chromatograms of samples prepared at pH 3, 5, and 7, when each sample was analyzed by HPLC immediately after preparation (data not shown). The results suggest that the carbinol pseudo-base or quinoidal-base was protonated to be reversibly the flavylium cation during HPLC analysis because the mobile phase was acidic (pH ∼2.5). Thus, we tried to examine the stability of acylated anthocyanins in red radish extract during storage at different pH values (3, 5, and 7) under light, heat, or H_2O_2.

Photostability. The stability of red radish extract to light was examined at different pH values (3, 5, and 7). The photostability

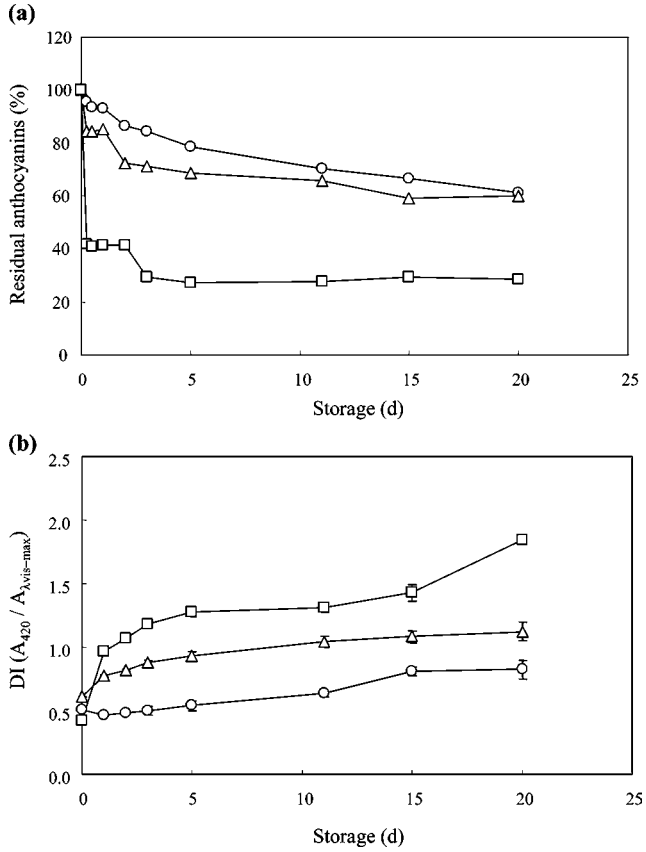


Figure 4. Changes in absorbance at $\lambda_{vis-max}$ (a) and degradation index (b) of red radish extract during storage under light at different pH values. pH 3: ○, pH 5: △, and pH 7: □.

of samples depended on the pH of the solution (**Figure 4**a). At pH 3 and 5 more than 60% of anthocyanins were retained after 20 d light exposure (480 h), whereas at pH 7 anthocyanin content reduced to 40% within 1 d. It is reported that the DI value indicates the proportion of degraded anthocyanin in a

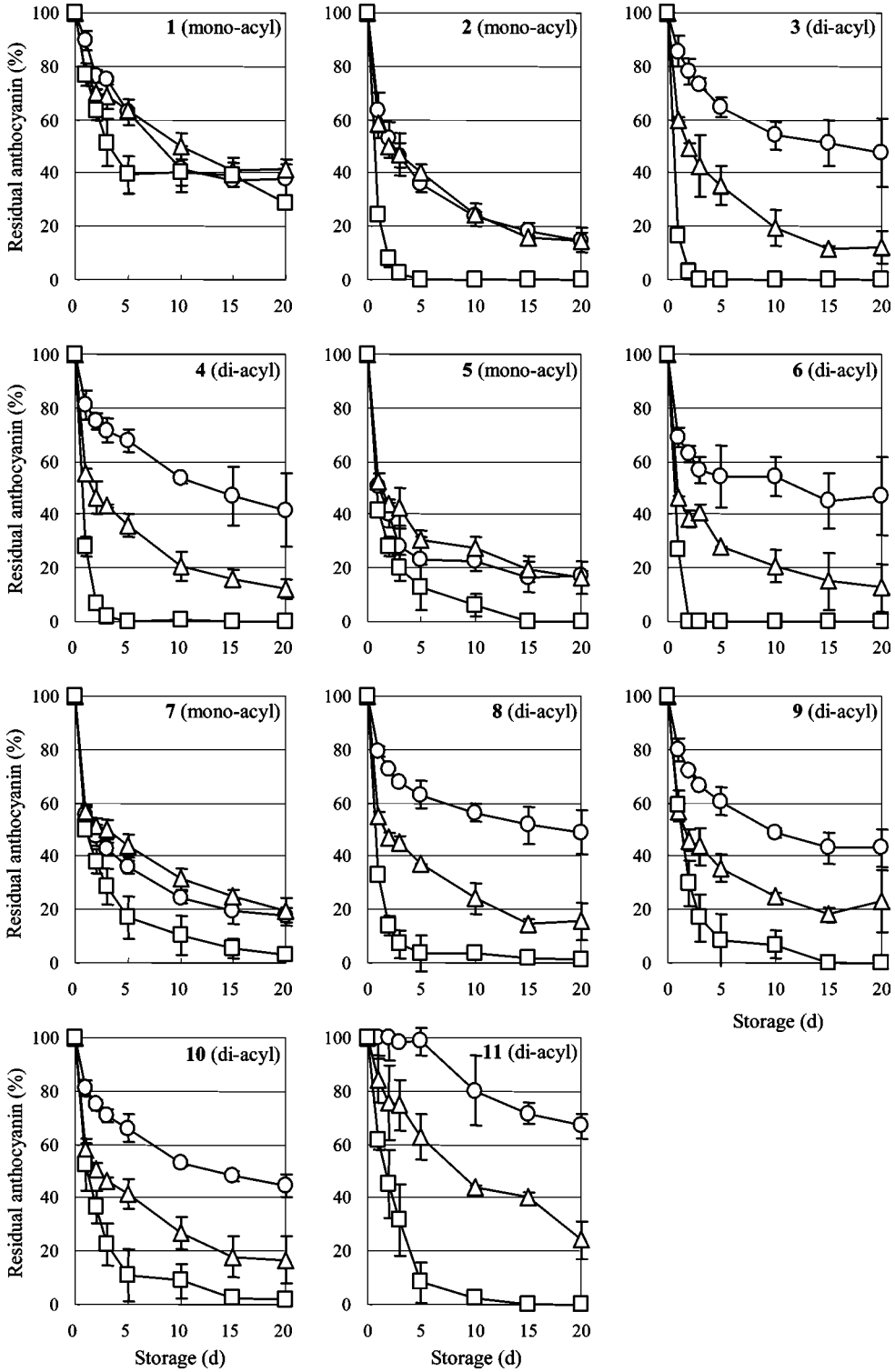


Figure 5. Changes in acylated anthocyanins in red radish extract during storage under light at different pH values. pH 3: ○, pH 5: △, pH 7: □.

sample (*29*). As shown in **Figure 4b**, DI values of samples increased during light exposure, depending on the pH. The results also suggested that the anthocyanins were more stable to light at lower pH than at higher pH. On the other hand, HPLC analyses revealed that the photostability of individual acylated anthocyanins were quite different. The degradation behavior and initial degradation rates of acylated anthocyanins in samples exposed to light are shown in **Figure 5** and **Table 1**, respectively. The photostability of monoacylated anthocyanins **1**, **2**, **5**, and **7** was independent of the pH between pH 3 and 5. The diacylated anthocyanins such as compounds **3**, **4**, **6**, **8**, **9**, **10**, and **11** were significantly more stable at pH 3 than the monoacylated anthocyanins ($p < 0.05$). The diacylated anthocyanins contribute to the high stability of red radish extract at pH 3. On the other hand, no significant difference in initial degradation rates of acylated anthocyanins other than compound **1** was observed at pH 5, whereas compounds **1**, **5**, **7**, **9**, **10**, and **11** were more stable at pH 7 than **2**, **3**, **4**, and **6**. Compounds **1**, **5**, **7**, **9**, **10**, and **11** are anthocyanins acylated with *p*-coumaric or ferulic acids, whereas the compounds **2**, **3**, **4**, and **6** are acylated anthocyanins with caffeic acids. Thus, these results suggest that the number of intramolecular acyl units affect the

Table 1. Initial Degradation Rate of Acylated Anthocyanins in Red Radish Extract

	pH 3	pH 5	pH 7
		Light (%/d)	
1	−8.1 ± 1.0	−10.5 ± 1.2	−12.5 ± 3.1
2	−18.3 ± 1.6	−19.1 ± 2.2	−29.7 ± 1.7
3	−9.4 ± 0.9	−17.9 ± 2.9	−32.6 ± 1.9
4	−7.2 ± 1.7	−18.7 ± 1.8	−32.0 ± 0.7
5	−22.4 ± 4.5	−16.6 ± 5.3	−23.9 ± 1.9
6	−13.2 ± 2.1	−20.6 ± 2.9	−34.5 ± 2.6
7	−18.9 ± 1.9	−17.4 ± 1.9	−21.0 ± 2.4
8	−10.1 ± 0.4	−18.6 ± 1.5	−28.5 ± 1.8
9	−9.5 ± 0.5	−16.7 ± 2.3	−23.2 ± 4.1
10	−9.8 ± 0.4	−17.9 ± 1.3	−17.2 ± 9.8
11	−0.5 ± 0.0	−15.2 ± 6.0	−17.0 ± 8.3
		Heat (%/h)	
1	−11.7 ± 0.3	−17.7 ± 1.5	−23.4 ± 2.2
2	−12.7 ± 0.5	−17.4 ± 0.4	−25.0 ± 0.0
3	−8.5 ± 2.1	−18.0 ± 0.9	−25.0 ± 0.0
4	−10.0 ± 1.6	−18.8 ± 0.9	−25.0 ± 0.0
5	−13.3 ± 0.4	−17.3 ± 1.0	−24.0 ± 1.4
6	−10.1 ± 0.8	−17.5 ± 0.5	−25.0 ± 0.0
7	−12.5 ± 0.5	−17.2 ± 0.9	−23.9 ± 1.6
8	−10.4 ± 0.7	−17.6 ± 0.8	−25.0 ± 0.0
9	−10.0 ± 0.8	−15.7 ± 1.1	−23.9 ± 1.6
10	−10.6 ± 0.8	−16.2 ± 1.1	−23.8 ± 1.6
11	−9.1 ± 1.2	−19.8 ± 2.6	−25.0 ± 0.0
		H_2O_2 (%/h)	
1	−10.7 ± 0.4	−7.2 ± 1.2	−8.6 ± 0.4
2	−8.9 ± 0.1	−4.7 ± 0.9	−7.8 ± 0.7
3	−5.3 ± 0.2	−6.5 ± 0.6	−8.5 ± 0.2
4	−4.2 ± 0.5	−5.3 ± 0.6	−8.7 ± 0.4
5	−8.5 ± 0.3	−3.5 ± 1.2	−7.1 ± 0.8
6	−6.2 ± 1.1	−5.1 ± 0.5	−7.3 ± 0.5
7	−8.9 ± 0.3	−4.4 ± 0.6	−6.9 ± 0.5
8	−5.2 ± 0.1	−5.0 ± 0.7	−6.3 ± 0.5
9	−4.7 ± 0.7	−5.3 ± 0.6	−5.9 ± 0.5
10	−4.1 ± 0.5	−4.3 ± 0.7	−4.7 ± 0.4
11	−6.8 ± 0.5	−6.5 ± 1.0	−13.9 ± 0.7

stability of anthocyanins to light at pH 3, whereas the characteristics of intramolecular acyl units affect the stability at pH 7.

Thermostability. During heating, the color of red radish anthocyanin extracts disappeared and/or changed to yellow. The degradation behavior of samples at pH 3, 5, and 7 were similar to that of light, although the DI values increased even more during heating (**Figure 6a** and **6b**). The results suggested the increase of browning (A_{420}) during heating. The degradation behavior and initial degradation rates of individual acylated anthocyanins in samples at pH 3, 5, and 7 are shown in **Figure 7** and **Table 1**, respectively. At pH 3, the diacylated anthocyanins were significantly more stable to heat than monoacylated anthocyanins, as well as those to light, suggesting that the number of intramolecular acyl units affect the heat stability. Although no significant difference was observed, compounds **1**, **5**, **7**, **9**, and **10**, anthocyanins acylated with *p*-coumaric or ferulic acids, were a little more stable than anthocyanins acylated with caffeic acids.

Stability to Hydrogen Peroxide. The degradation behavior of red radish extract to H_2O_2 was almost the same as that of light and heat, depending on the pH of the solution. In addition, the stability depended on the concentration of H_2O_2 (**Figure 8a** and **8b**). **Figure 9** and **Table 1** show the degradation behavior and initial degradation rates of individual acylated anthocyanins in samples to H_2O_2 at different pH values, respectively. The behavior was greatly different from those of light and heat, and it was found that the stability of some acylated anthocyanins, particularly monoacylated anthocyanins to H_2O_2, was independent of the pH of the solution. At pH 3, diacylated anthocyanins were significantly more stable than monoacylated anthocyanins. Interestingly, similar degradation patterns were obtained for compounds **2**, **5**, and **7**, for **3** and **4**, and for **6**, **8**, **9**, and **10**. Compounds **2**, **5**, and **7** are anthocyanins monoacylated with caffeic, *p*-coumaric, and ferulic acids in the R1-position, respectively, whereas **1** has the ferulic acid in R3-position (**Figure 2**). No significant difference in degradation behavior of compounds **2**, **5**, and **7** was observed, suggesting that characteristics of the acyl unit in monoacylated anthocyanins have no effect on the stability. However, compounds **2**, **5**, and **7** were significantly more stable than **1** at pH 3 and 5, suggesting that the binding site of the intramolecular acyl unit would affect the stability. The result that compound **10** was more stable than **11** also supported this suggestion. For diacylated anthocyanins, compounds **9** and **10** were significantly more stable than **3** and **4** at pH 7, although there was no significant difference in degradation rates at pH 3. Anthocyanins diacylated with *p*-coumaric or ferulic acids would be more stable than ones diacylated with caffeic acids at higher pH. Thus, these results suggest that the number of intramolecular acyl units affect the stability to H_2O_2 at lower pH, whereas the binding sites and the characteristics of intramolecular acyl units affect the stability at higher pH.

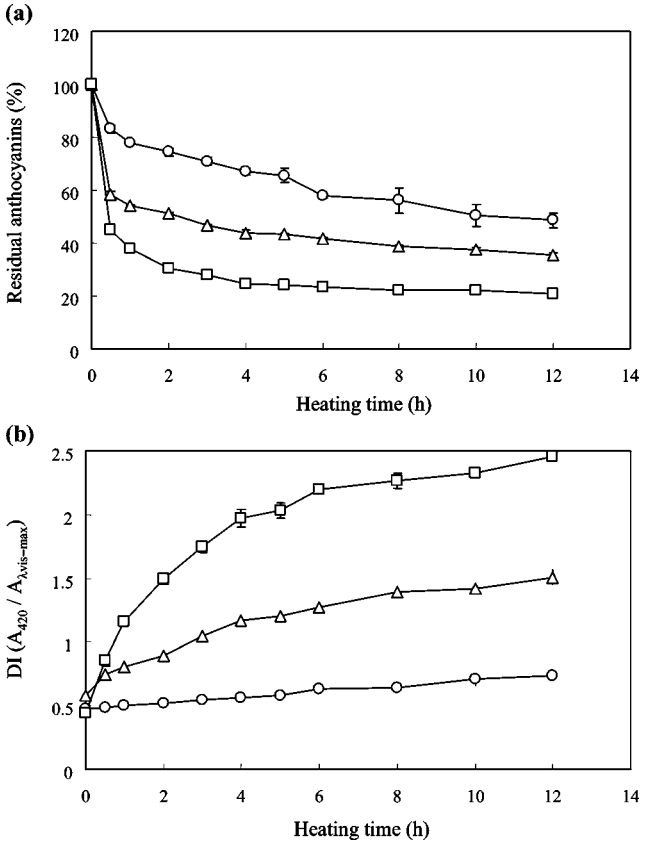


Figure 6. Changes in absorbance at $\lambda_{vis-max}$ (a) and degradation index (b) of red radish extract during heating at different pH values. pH 3: ○, pH 5: △, and pH 7: □.

Changes in Anthocyanin Profile during Storage. Typical HPLC patterns of samples during storage under light, heat, and H_2O_2 at pH 3 are shown in **Figure 10**. When the sample was exposed to light, the strength of seven unidentified peaks increased remarkably, compared with those of untreated red radish extract (**Figure 1**). At pH 5 and 7, the strength of peaks increased a little (data not shown). On the other hand, three increasing peaks were observed from heat treatment. Judging

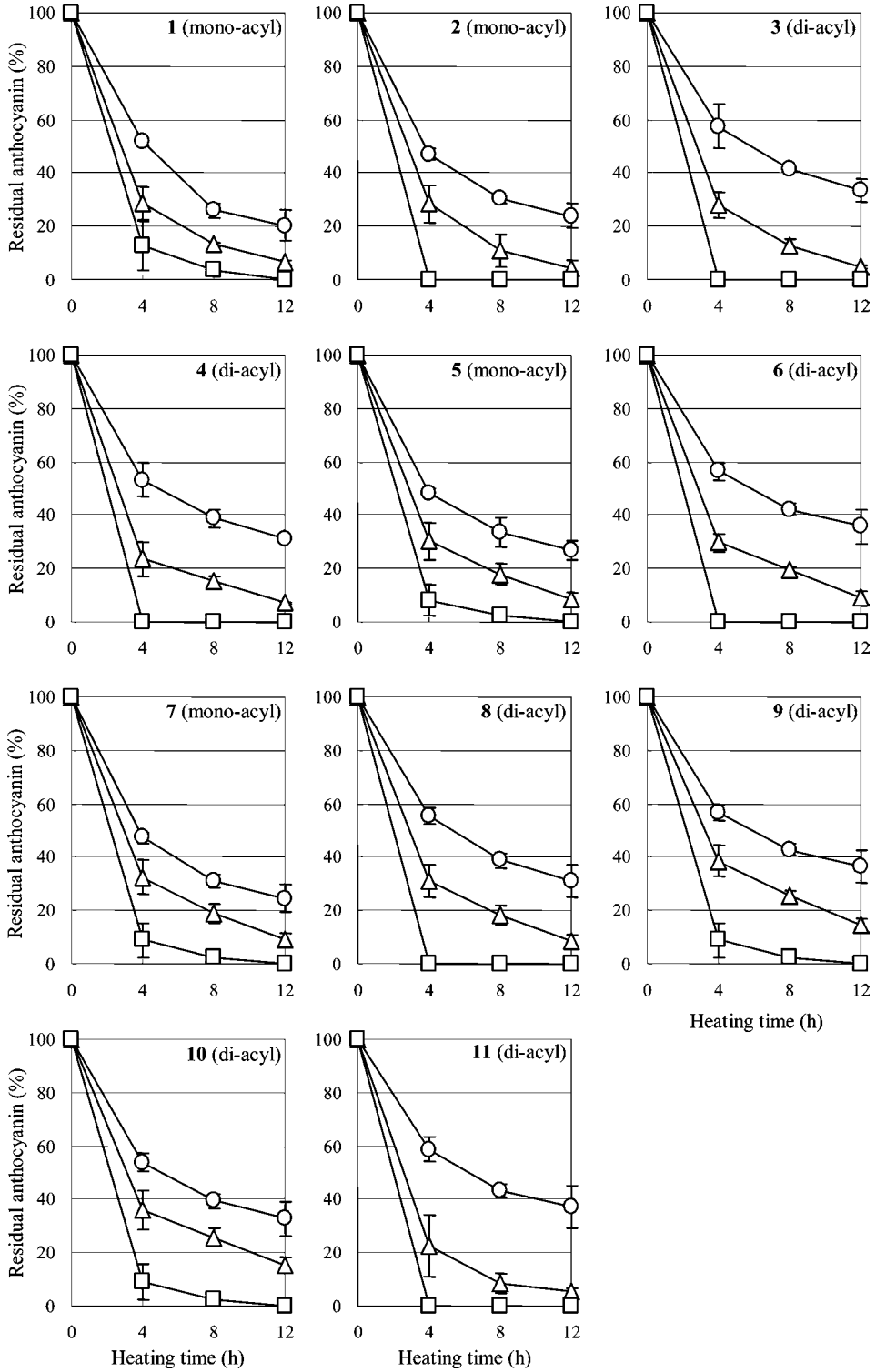


Figure 7. Changes in acylated anthocyanins in red radish extract during heating at different pH values. pH 3: ○, pH 5: △, pH 7: □.

from the detection at 520 nm, they might be *Z*-isomer, deglycosyl, or deacyl forms of acylated anthocyanins. Interestingly, some of the peaks existed in untreated red radish extract. The results imply that some unidentified anthocyanins might form during the manufacturing process of the extract. No increasing peak was observed from H_2O_2 treatment. The results suggest that the degradation mechanism of acylated anthocyanins under light, heat, and H_2O_2 differs greatly.

DPPH Radical Scavenging Activity. The stability (reactivity) to hydrogen peroxide implies antioxidant ability against reactive oxygen species (ROS). In this study, DPPH radical scavenging activity of acylated anthocyanins at pH 3 and 7 was examined, using isolated nine acylated anthocyanins, **1**, **2**, **3**, **4**, **5**, **7**, **8**, **9**, and **10**. As shown in **Figure 11**,all acylated anthocyanins showed significantly stronger antioxidant activity than pelargonidin (Pg) and pelargonidin-3-glucoside (Pg-3-G). In addition, all acylated anthocyanins showed similar antioxidant activity at pH 7 to those at pH 3, whereas the activity of pelargonidin and pelargonidin-3-glucoside lowered remarkably at pH 7, because of the low stability of anthocyanins. On the other hand, the activities of acylated anthocyanins were greatly different from the characteristics of intramolecular acyl units,

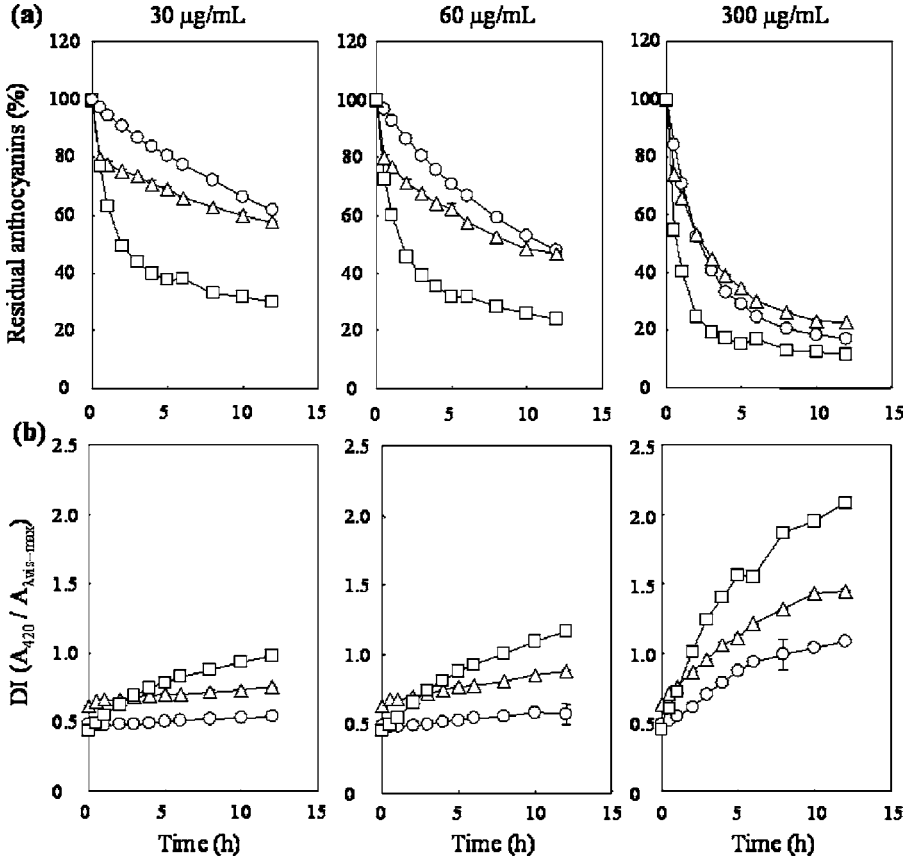


Figure 8. Changes in absorbance at $\lambda_{vis-max}$ (a) and degradation index (b) of red radish extract at different H_2O_2 concentrations and pH. pH 3: ○, pH 5: △, and pH 7: □.

depending on the activity of binding cinnamic acids in the R1-position (IC_{50} values of cinnamic acids: caffeic acid (0.25 mmol/L) > ferulic acid (0.77 mmol/L) > *p*-coumaric acid (3.69 mmol/L), data not shown). Interestingly, compound **4**, anthocyanin acylated with caffeic and ferulic acids in the R1- and R3-positions, respectively, had higher activity than **8** with ferulic and caffeic acids in R1- and R3-positions, respectively. Also, compound **1**, anthocyanin acylated with ferulic acid in R3-position had activity higher than **7** with ferulic acid in R1-position. These results suggest that the binding site of intramolecular acyl units highly affect antioxidant activity.

DISCUSSION

Many anthocyanins were present in red radish extract. The major anthocyanins were pelargonidin-3-sophoroside-5-glucoside with a combination of *p*-coumaric, ferulic, and caffeic acids. The extract was more stable to light, heat, and H_2O_2 at lower pH, depending on the pH of the solution. On the other hand, the stability of individual acylated anthocyanins in the extract varied considerably under different conditions. The number of the intramolecular acyl units contribute to the stability at lower pH, whereas the characteristics of the intramolecular acyl units influence the stability at higher pH.

The acylation makes the anthocyanins more stable through intramolecular copigmentation, protecting the flavylium chromophore from the nucleophilic attack of water. It is suggested that diacylated anthocyanins might be more stable than monoacylated ones due to a sandwich type stacking by hydrophobic interactions between the planar aromatic residues of intramolecular acyl units and the flavylium nucleus (*9*, *12*). Yoshida et al. (*29*) have reported that an *E*-isomer of monoacylated anthocyanin was isomerized to the *Z*-isomer under UV irradiation, but in diacylated anthocyanins the isomerization from *E*-isomer to *Z*-isomer was suppressed, and they concluded that the stability of anthocyanins to light highly depends on the molecular stacking. Our results for the high stability of diacylated anthocyanins to light and heat at pH 3 (**Figure 5** and **7** and **Table 1**) also support this proposal. On the other hand, at pH 5 there was no difference of stability between mono- and diacylated anthocyanins, and at pH 7 the characteristics of intramolecular acyl units, not the number, affected the stability. Thus, these results suggest that the acyl units in the molecules might be away from the flavylium nucleus, as the pH of the solution is neutral and the space position of each acyl unit might differ in intramolecular stacking conformation. The stability of anthocyanins with different acyl units at different pH values (acidic to neutral pH) has been scarcely investigated in detail.

The difference in chromatograms of red radish extract under light, heat, and H_2O_2 suggests that degradation mechanism of acylated anthocyanins differ (**Figure 10**). Also, the results suggest that some unknown anthocyanins were formed by photo- and heat-degradation, judging from detection at 520 nm. It is known that opening of the heterocycle (C-ring) and then formation of chalcone occur as the first degradation step of anthocyanidin. For acylated anthocyanin, isomerization of intramolecular acyl units of acylated anthocyanins (monoacylated anthocyanin) under light has been reported (*29*, *30*). The deglycosylation and hydrolysis of acylated anthocyanin under heat led to formation of the corresponding anthocyanin glycosides and anthocyanidin, followed by degradation products from anthocyanidin (*31*). Further investigation such as identification of unknown anthocyanins is needed to clarify the degradation mechanism of acylated anthocyanin.

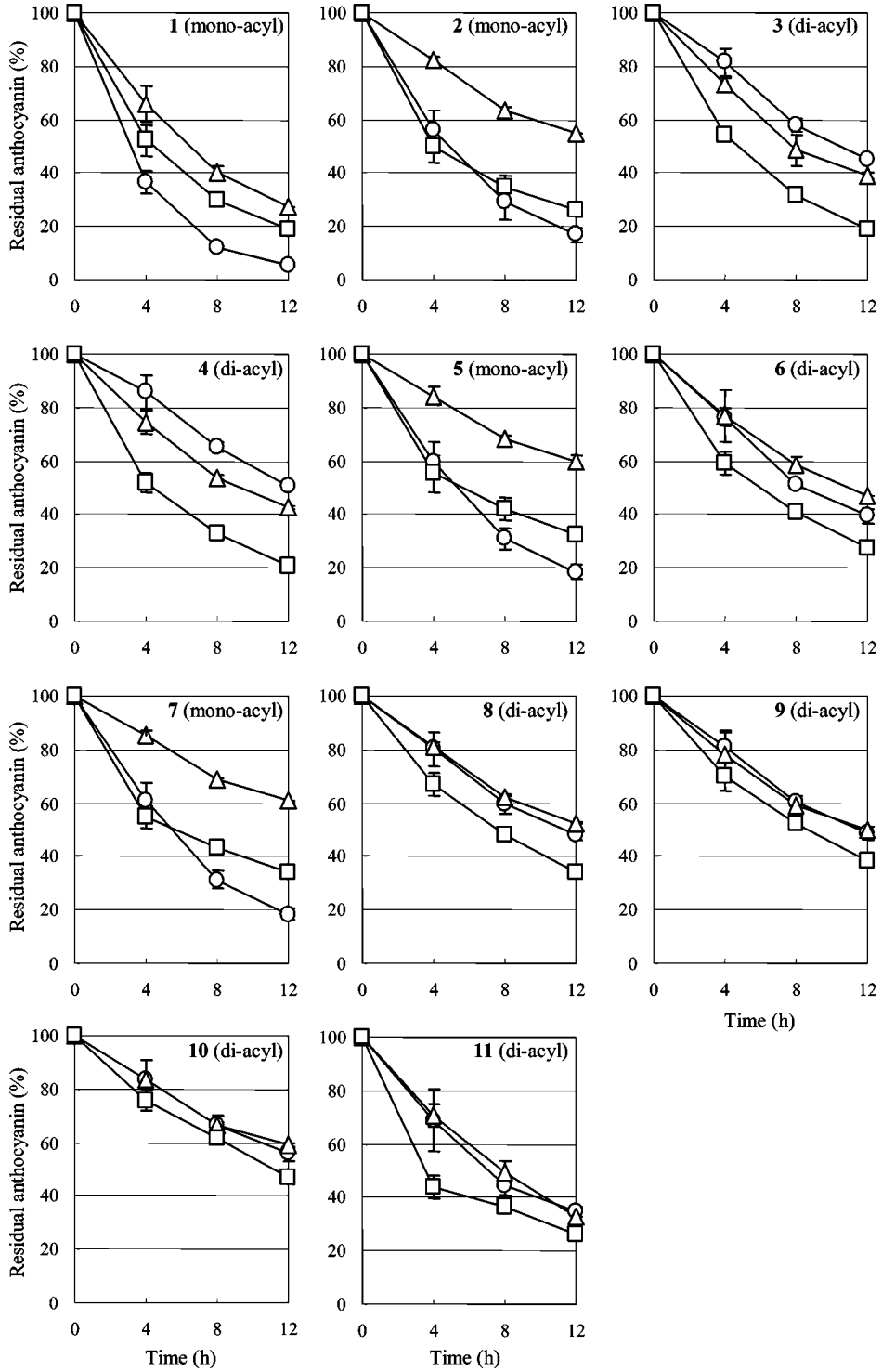


Figure 9. Changes in acylated anthocyanins in red radish extract in the presence of 60 μg/mL of H_2O_2 at different pH values. pH 3: ○, pH 5: △, pH 7: □.

Antioxidant activity of acylated anthocyanins was also affected by the characteristics of intramolecular acyl units. Özkan et al. (*32*) have reported two factors for the degradation of anthocyanins by H_2O_2: (a) free radicals and HOO^- anion formed by the decomposition and dissociation of H_2O_2 and (b) quinines formed the H_2O_2-catalyzed oxidation of phenolic compounds. At pH 3, degradation rates of monoacylated anthocyanins were faster than those of diacylated anthocyanins (**Figure 9** and **Table 1**). The results suggest a high reactivity of monoacylated anthocyanins to H_2O_2. Monoacylated anthocyanins suppress oxidation induced by H_2O_2 more than diacylated anthocyanins at lower pH. In our previous reports (*33*), degradation rates of compounds **4** and **8** with peroxyl radicals were faster than that of **10**. However, the degradation rates (reactivity) of acylated anthocyanins were not in accord with their DPPH radical scavenging activity. The results and findings suggest that the antioxidant capacity of acylated anthocyanins depends on the characteristics of the radicals. For antioxidant capacity of acylated anthocyanins, estimation by several antioxidant methods is needed. Measurement of antioxidant capacity with peroxyl and hydroxyl radicals is currently in progress.

The characteristics of intramolecular acyl units highly affected the stability and antioxidant activity. The anthocyanins diacylated with *p*-coumaric or ferulic acids tended to be stable to

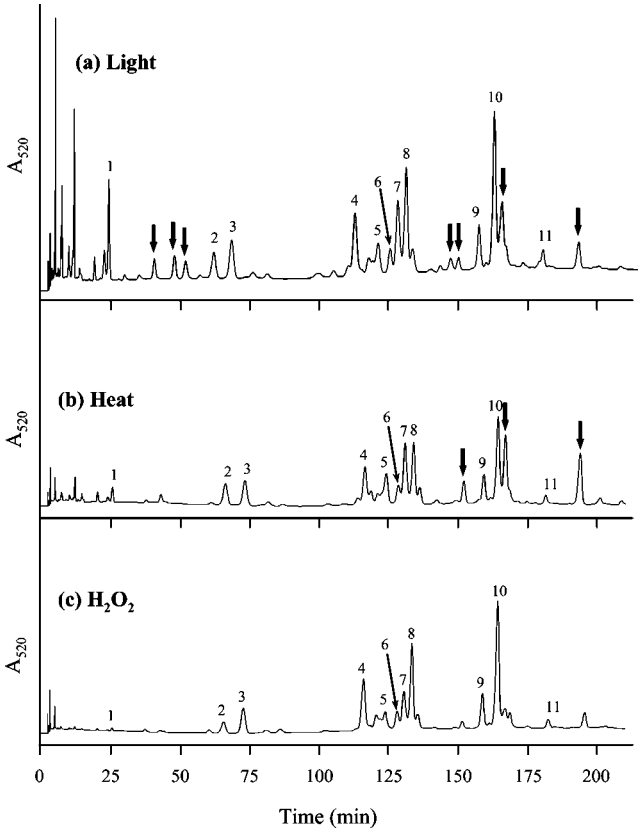


Figure 10. Typical HPLC profiles of red radish extract during storage (a) after 5 days under light at pH 3, (b) after 8 h under heat at pH 3, and (c) after 8 h under H_2O_2 at pH 3. The arrows indicate the peak for which intensity increased compared to those of the untreated red radish extract.

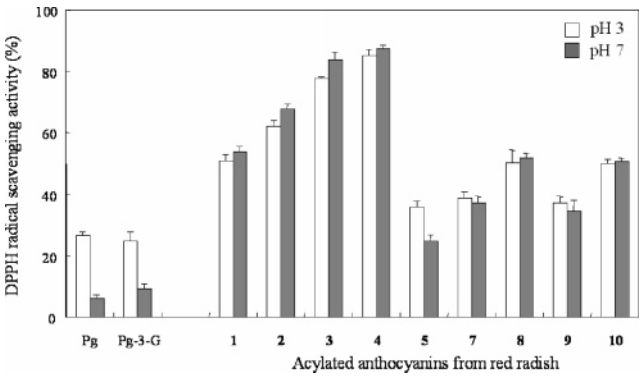


Figure 11. DPPH radical scavenging activity of acylated anthocyanin from red radish. Each value is the mean ± SD ($n = 5$). Pg, pelargonidin; Pg-3-G, pelargonidin-3-glucoside.

light, heat, and H_2O_2, whereas the ones diacylated with caffeic acid tended to have high antioxidant activity, although the binding site of intramolecular acyl units have a very important impact. Terahara et al. (*34*) have reported that cyanidin acylated with caffeic and *p*-coumaric acids was a little more stable in neutral aqueous solution and had higher antioxidant activity than cyanidin acylated with two *p*-coumaric acids. In addition, peonidin acylated with two *p*-coumaric acids was more stable but had lower antioxidant activity than cyanidin acylated with two *p*-coumaric acids. These findings imply that the characteristics of aglycone and intramolecular acyl units also affect the stability and antioxidant activity.

Anthocyanins in foods undergo severe physical and chemical treatments during food processing. Pelargonidin and pelargonidin-3-glucoside were degraded within 1 h in all experiments in this study (within a few minutes at pH 7; data not shown). Although acylated anthocyanins show higher stability and antioxidant activity than the corresponding anthocyanidins and their glycosides, the characteristics, the number, the binding site, and probably the spacial position of intramolecular acyl units affect the stability and antioxidant activity of acylated anthocyanins. There are several reports for the effects of other ingredients such as sugar, ascorbic acid, and phenolic compounds in food on the stability of anthocyanins (*27*, *30*, *35*). Screening of anthocyanins based on their chemical structure might be needed to apply them effectively in the food system, particularly at neutral pH.

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