JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enhanced Photostability of Monascus Pigments Derived with Various Amino Acids via Fermentation

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The photostability of 18 amino acid derivatives from monascus pigment was tested under various physical and chemical conditions. Under sunlight, the half-life of derivatives was increased to 1.45−5.58 h, corresponding to a 6−25-fold improvement over a control red pigment (0.22 h). The degradation of pigment derivatives followed a first-order reaction, and the pigment stability increased with an increasing concentration while it decreased with both an increase and decrease in pH from 7. The stabilities of derivatives decreased in descending order in hexane, ethanol, propanol, methanol, ethyl ether, distilled water, chloroform, and acetonitrile. Pigment stability under UV light (365 nm) showed a pattern similar to stability after exposure to sunlight. After 30 days of incubation at 30 °C, more than 80% of the initial derivative contents remained while only 29% of the control red remained. The differences in degradation patterns that control red gradually changed to brown whereas the phenylalanine derivative remained a weak red were confirmed by HPLC analysis.

KEYWORDS: Monascus; pigment; amino acid derivatives; photostability; kinetics

INTRODUCTION

Species of genus *Monascus* have been used for many years as a colorant for wines and bean curds and as a medicine in East-Asian countries (*1*). The pigments produced by *Monascus* species consist of six major compounds including the yellows of monascin (*2*−*4*) and ankaflavin (*5*), oranges of monascorubrin (*6*, *7*) and rubropunctatin (*8*), and reds of monascorubramine (*9*) and rubropunctamine (*10*). The orange compounds readily react with compounds containing amino groups in ethanol, via a ring-opening and a Schiff rearrangement (*11*, *12*). Monascus pigments are good food colorants because they have a variety of properties, including different colors of yellow to red (*13*−*15*). They are stable in a pH range of 2−10 and heat-stable to autoclaving. However, their low water solubility and decoloring under light have limited the use of monascus pigments in the food industry (*15*). There is a report (*16*) that monascus pigments are more sensitive to UV than to other wavelengths. According to Jung et al. (*17*), various red colors can be produced by addition of 20 individual amino acids as a precursor during *Monascus* cultivation. The degree of hydrophobicity/hydrophilicity of monascus pigments can be regulated, and these pigments have the advantage of being easily and cheaply produced on an industrial scale. There are a few reports (*15*, *16*) that monascus pigments are sensitive to pH, UV, and temperature. According to our preliminary analyses, the pigments are very sensitive to sunlight and are decolored quickly. However, there are few studies focused on the enhancement of photostability of various pigment derivatives. The photostability of monascus pigments must be improved for wide industrial application.

In this study, we produced derivatives of monascus pigments from fermentation with addition of various amino acids. The pigment photostability was analyzed under varying conditions of pH, solvent addition, and temperature.

MATERIALS AND METHODS

Reagents. A glucose−peptone medium and an N-source-modified medium were purchased from Difco Co. Amino acids were products of Sigma-Aldrich Co. Chloroform and methanol were obtained from Tedia Co., and other chemicals were products of Duksan Pure Chemical Co.

Cultivation for Synthesis of Derivatives. The strain used for production of pigments was *Monascus* sp. KCCM 10093 (KCCM: Korea Culture Center for Microorganisms) maintained on a slant. Cultivations were performed as previously described (*17*).

Extraction of Pigments. After 100 mL of fermentation broth was placed in 500 mL flasks, 100 mL of ethyl alcohol was added. The flasks were incubated on a reciprocal shaker (International Science Co.) at 30 °C and 180 rpm. The solutions were then filtered to obtain crude pigment extracts.

Purification of Pigment Derivatives. Pure pigment solutions were obtained by collecting major pigment spots on prep-TLC (*17*). The absorbance values at 500 nm were measured using a UV−vis spectrometer (Shimadzu Co., UV-1201, Tokyo).

Photostability Tests of Pigment Derivatives. Glass vials containing pigments in ethanol whose initial concentrations were adjusted to 2.0 ± 0.5 OD units at 500 nm were exposed to sunlight. Data for sunlight irradiation intensity/h during the experimental period were given everyday by the Korea Meteorological Administration. Photostability

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10.1021/jf0510283 CCC: $30.25
Published on Web 08/13/2005

Table 1. Kinetic Data for Decoloration of Various Pigment Derivatives in Ethanol under Sunlight Irradiation ($R^2 > 0.99$)[a]

amino acid moiety	k (h^{-1})	$t_{1/2}$ (h)	amino acid moiety	k (h^{-1})	$t_{1/2}$ (h)
control red	3.200 ± 0.105	0.22 ± 0.01	phenylalanine	0.159 ± 0.001	4.36 ± 0.02
asparagine	0.125 ± 0.009	5.58 ± 0.41	aspartic acid	0.172 ± 0.016	4.05 ± 0.38
tyrosine	0.131 ± 0.005	5.28 ± 0.21	cysteine	0.182 ± 0.001	3.77 ± 0.02
alanine	0.134 ± 0.019	5.25 ± 0.74	leucine	0.243 ± 0.004	2.86 ± 0.05
threonine	0.137 ± 0.003	5.07 ± 0.12	methionine	0.282 ± 0.011	2.46 ± 0.10
glycine	0.137 ± 0.001	5.06 ± 0.02	lysine	0.290 ± 0.016	2.39 ± 0.55
isoleucine	0.130 ± 0.005	5.03 ± 0.20	arginine	0.322 ± 0.007	2.15 ± 0.21
glutamic acid	0.140 ± 0.004	4.96 ± 0.15	histidine[b]	0.478 ± 0.005	1.45 ± 0.14
tryptophan	0.152 ± 0.008	4.57 ± 0.25	Lac	0.026 ± 0.007	26.50 ± 1.87
serine	0.158 ± 0.006	4.38 ± 0.15	cochineal	0.138 ± 0.018	5.02 ± 0.81
valine	0.158 ± 0.004	4.38 ± 0.12			

[a] The average intensity of sunlight irradiation/h during the experimental period was 2.30 ± 0.20 MJ/m^2. [b] R^2 of histidine derivative was 0.97.

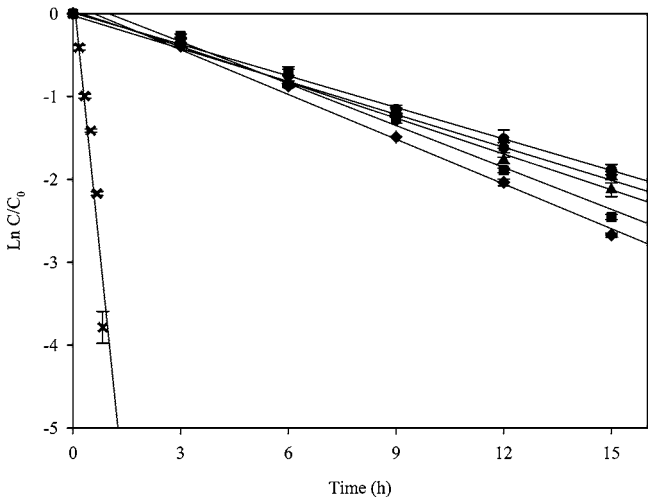

Figure 1. Semilogarithmic plots of decoloration of various pigment derivatives under sunlight irradiation ($R^2 > 0.99$ for all the pigments): ×, control red pigment; ●, asparagine; ▲, glycine; ■, isoleucine; ◆, phenylalanine; ⬟, threonine; Co = initial pigment concentration; C = pigment concentration at *t*. The average intensity of sunlight irradiation/h during the experimental period was 2.30 ± 0.20 MJ/m^2.

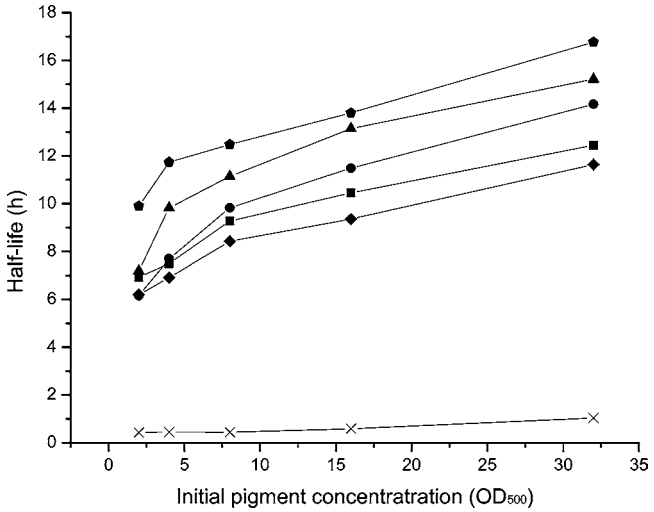

Figure 2. Change in half-life ($t_{1/2}$) of pigment derivatives with the concentration level under sunlight irradiation: ×, control red pigment; ●, asparagine; ▲, glycine; ■, isoleucine; ◆, phenylalanine; ⬟, threonine. The average intensity of sunlight irradiation/h during the experimental period was 2.70 ± 0.79 MJ/m^2.

was tested only for 2−3 h between 11 a.m. and 3 p.m. a day when the intensity was the highest. This test continued for 4−6 days until the pigment concentration reached 10−20% of initial values. All the

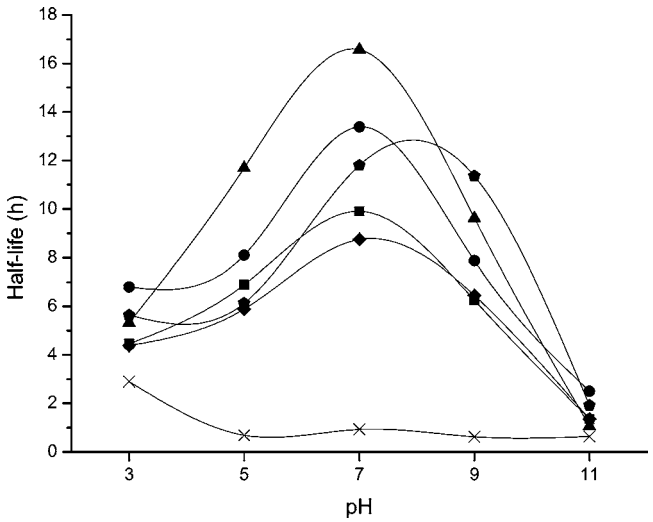

Figure 3. Effect of pH on the half-life of pigment derivatives in buffer solutions under sunlight irradiation: ×, control red pigment; ●, asparagine; ▲, glycine; ■, isoleucine; ◆, phenylalanine; ⬟, threonine. The average intensity of sunlight irradiation/h during the experimental period was 3.13 ± 0.80 MJ/m^2.

samples in a one-set experiment were tested under identical conditions for pigment concentration, sunlight irradiation, and experimental period. But the conditions were varied on different sets of experiments Alternatively, a UV lamp (365 nm, 8W, Vilber Lourmat, VL-4-LC, Marne La Vallee, France) was used to irradiate pigment solutions in a black box. Qualitative and quantitative analyses of pigments were performed with HPLC. The HPLC system(Waters, Milford, MA) consisted of a BDS C_{18} column (250 × 4.6 mm, 5μm i.d., Hypersil), a W2690 separation module, a W2487 UV−vis detector, and a Millenium software data module. Operational conditions were a run time of 40 min, a fluid velocity of 0.8 mL/min, and an elution gradient of distilled water/methanol of 100:0 to 10:90. Detections were carried out at 500 nm. The photostability of control red, an original product of fermentation, was compared with the amino acid derivatives of the pigments. The original red pigments (monascorubramine and rubropunctamine), which were produced by *Monascus* species under no adding condition of amino acids, were named as "control red". The photostability of cochineal and Lac pigments, well-known red food colorants, was also tested.

Procedures for Kinetic Analysis. First-order reactions were assumed for pigment decomposition as follows: $dC/dt = -kC$. The equation was integrated as follows: $\ln(C/C_0) = -kt$ (C is the pigment concentration at t, C_0 the pigment concentration at $t = 0$, k the rate constant (min^{-1}), and t the irradiation time (min)). The decomposition rate constants for all the pigments were estimated by plotting $\ln C/C_0$ with respect to t. Among them, five derivatives of asparagine, glycine, isoleucine, phenylalanine, and threonine were only plotted.

Effect of Varying Conditions on the Pigment Stability. The pigment concentration for light stability tests was varied in a range

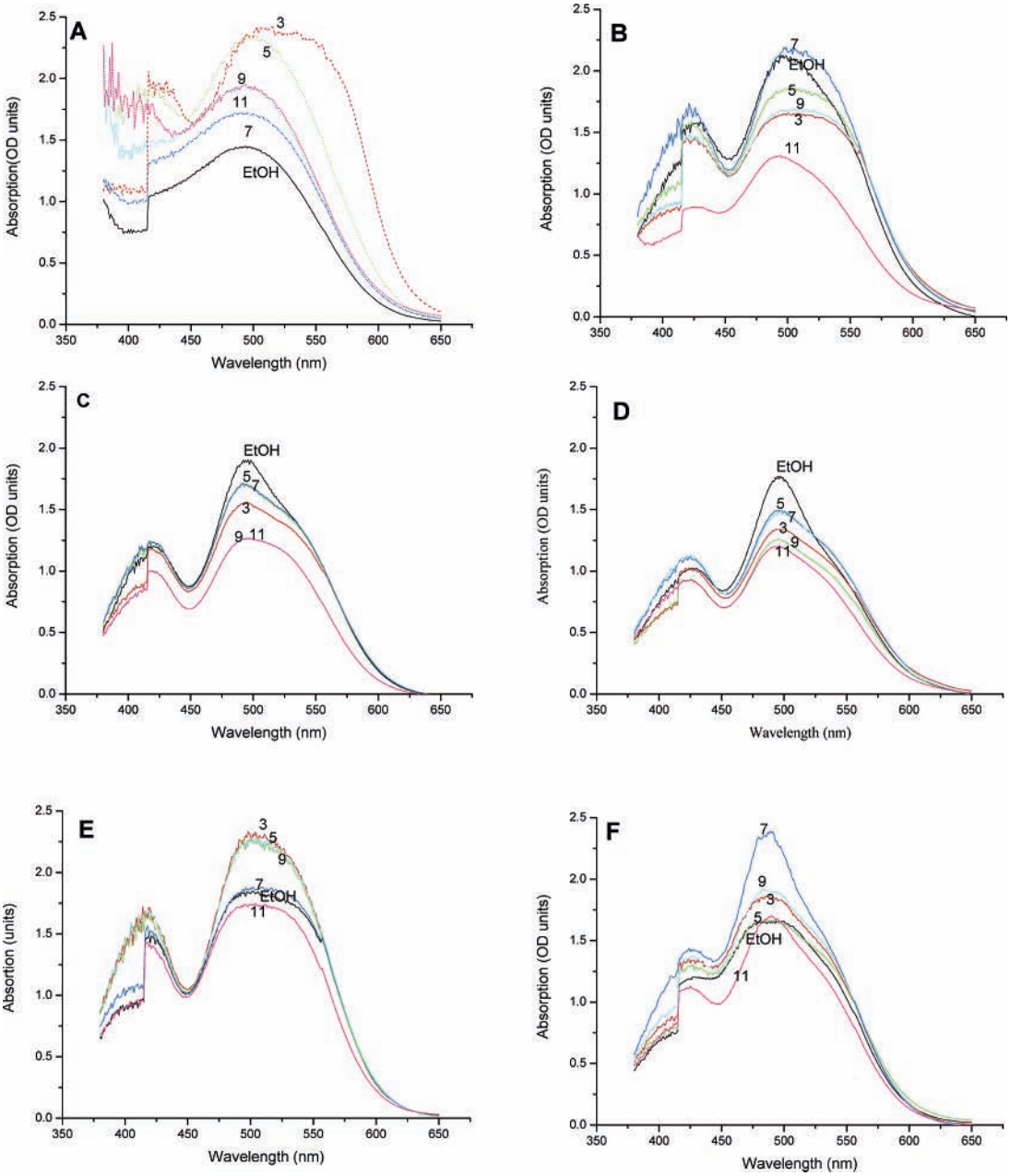

Figure 4. Spectral change of pigment derivatives in ethanol and various buffer solutions: A, control red pigments; B, asparagine; C, glycine; D, isoleucine; E, phenylalanine; F, threonine. The pigments were tested in ethanol and buffer solutions at pH 3, 5, 7, 9, and 11.

1.0−16.0 OD units in ethanol. Sample solution concentrations at 500 nm were measured using a spectrophotometer (Shimadzu Co., UV-1201, Tokyo, Japan) after the solutions were exposed to sunlight.

Buffer solutions (0.1 M, potassium phosphate) at 5 different pH values (3, 5, 7, 9, and 11) were prepared for pH stability tests. The solutions were mixed with pigment solutions at 1:1. After 6 h, the optical density of the pigment solutions was measured at 350−650 nm using a spectrophotometer (Beckman, DV 650, CA).

To investigate the effect of solvents on decoloring, pigments were dissolved in eight solvents with different polarity indices, including hexane (0.1), ethyl ether (2.8), propanol (3.9), chloroform (4.1), methanol (5.1), acetonitrile (5.8), ethanol, and deionized water (10.2).

The thermostability of pigments was tested after 30 days of incubation at temperatures of 4, 30, and 80 °C with no light irradiation. The pigment amounts remaining after treatment under various conditions were measured using HPLC.

Color Analysis of Pigments. After the concentration of pigment solutions was adjusted to 2.0 ± 0.5 OD units at 500 nm followed by incubation under various conditions, pigment color changes were measured using a colorimeter (Minolta Camera Co., CR-300, Osaka, Japan). The values of L^*, a^*, and b^* were estimated using a CIELAB color system and used to calculate the values of chroma (C) and hue angle (h_{ab}). The pigment degradation pattern was obtained from HPLC profiles at 250 nm.

Statistical Analysis. Decomposition rate constants were calculated via linear regression analysis. ANOVA tests for photostability data were performed using a SAS program (SAS Institute Inc., ver. 8.2). Then, Tukey's test was followed for comparison of means at a significance level of 0.05.

RESULTS AND DISCUSSION

Kinetic Analysis of the Photostability of Monascus Derivatives. Amino acid derivatives of monascus pigments were produced from cultivation of *Monascus* sp. KCCM10093. The photostabilities of 18 derivatives and control red were determined under sunlight. The decomposition process of all the pigments followed a first-order reaction. On the basis of this, the rate constants and half-life values ($t_{1/2}$) for pigments were

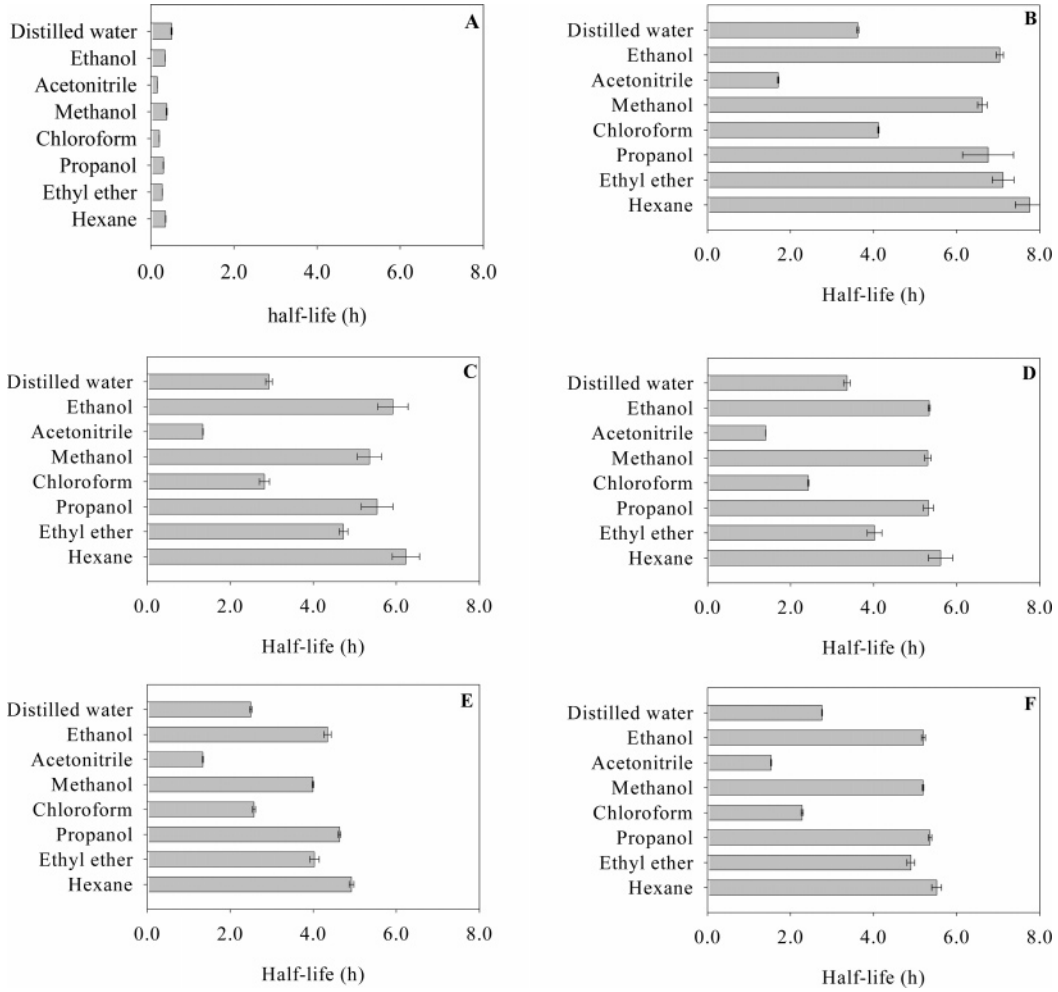

Figure 5. Half-life ($t_{1/2}$) of various pigment derivatives in different solvents under sunlight irradiation: A, control red pigments; B, asparagine; C, glycine; D, isoleucine; E, phenylalanine; F, threonine. The average intensity of sunlight irradiation/h during the experimental period was 1.65 ± 0.08 MJ/m^2.

Table 2. Photostability of Various Pigment Derivatives under UV Lamp (365 nm, 8W) Irradiation

amino acid moiety	k (h^{-1})	$t_{1/2}$ (h)
control red	0.029 ± 0.0011	24.02 ± 0.96
asparagine	0.003 ± 0.0003	278.93 ± 37.71
glycine	0.003 ± 0.0002	245.36 ± 17.72
isoleucine	0.004 ± 0.0002	192.64 ± 11.66
phenylalanine	0.004 ± 0.0000	167.84 ± 1.47
threonine	0.002 ± 0.0001	351.07 ± 24.92
cochineal	0.003 ± 0.0022	226.06 ± 8.19

estimated (**Table 1**) and semilogarithmic plots for only 6 out of 19 pigments were shown in **Figure 1**. The half-life of control red was 0.22 h. However, the half-life values of the amino acid derivatives were extended significantly to 1.45−5.58 h, corresponding to 6−25 times the life of control red. The derivatives were divided into 3 groups on the basis of the half-life. The most stable group consisted of derivatives of asparagine, tyrosine, alanine, threonine, glycine, and isoleucine moieties with half-life values of more than 5 h. The 2nd group included moieties of glutamic acid, serine, tryptophan, valine, and phenylalanine with half-life values of 4−5 h. The least stable group included moieties of aspartic acid, cysteine, leucine, methionine, lysine, arginine, and histidine with half-life values of less than 4 h. Thus, the stability of monascus pigment can be greatly improved by incorporation of amino acids. One-third of the derivatives had a high stability, similar to cochineal (**Table 1**), which is a common commercial red coloring agent.

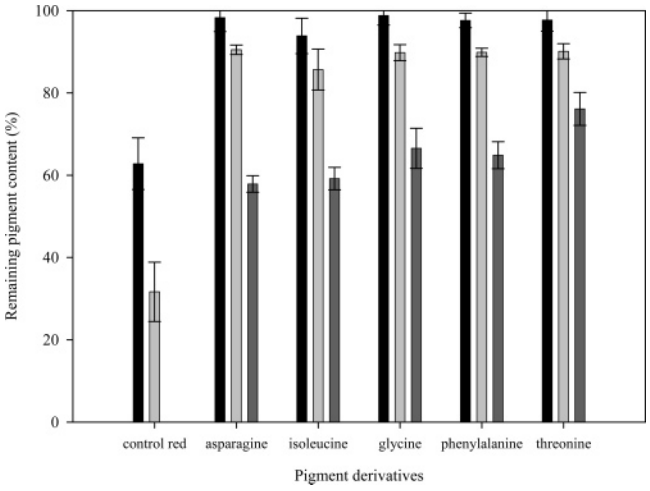

Figure 6. Remaining contents of various pigment derivatives after 30 days of storage at temperatures of 4 (black bars), 30 (light gray bars), and 80 °C (dark gray bars).

Control red and pretty stable derivatives of asparagine, glycine, isoleucine, phenylalanine, and threonine were chosen for stability testing. However, tyrosine and alanine derivatives were excluded because they are relatively similar in molecular structure to phenylalanine and glycine, respectively. Rate constant values for the decoloring reaction of pigment derivatives were estimated to be 0.125−0.478 h^{-1},

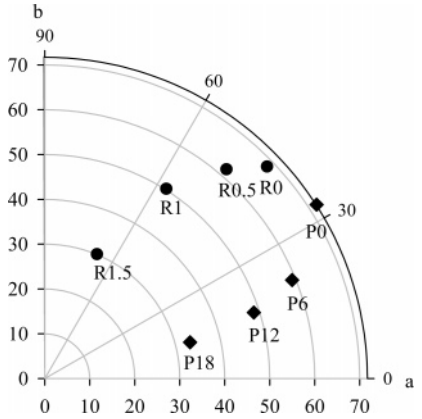

Figure 7. Scatter plot for the decoloration of control red and phenylalanine derivative under sunlight irradiation using a CIELAB colorimetric system. R = control red: R0, 0 h; R0.5, 0.5 h; R1, 1 h; R1.5, 1.5 h. P = phenylalanine derivative: P0, 0h; P6, 6 h; P12, 12 h; P18, 18 h. The average intensity of sunlight irradiation/h during the experimental period was 2.33 ± 0.26 MJ/m^2.

which is comparable to the control red value of 3.136 h^{-1} (**Table 1**).

Photostability with Varying Pigment Concentration. After the solutions of control red and the five amino acid derivatives were set to different concentrations (2.0, 4.0, 8.0, 16.0, and 32.0 OD units) in ethanol, they were exposed to sunlight. Similarly for all the pigments, their half-lives increased with increasing initial pigment concentration (**Figure 2**). In other words, high pigment photostabilities were obtained at high concentration levels. This phenomenon is supposedly due that the same amount of irradiation was applied under all experimental conditions so that the irradiation quantity/unit mass of pigment would be reduced with increasing pigment concentration.

Effect of pH on the Photostability of Pigments. After purified pigment derivatives were dissolved in ethanol, various pH buffers were added to the solution at a 1:1 ratio. The half-life of control red and the 5 derivatives with varying pH values was determined (**Figure 3**). Control red was most stable at pH 3 while the derivatives were most stable at pH 7. The half-life of derivatives decreased with both an increasing and a decreasing pH value from 7 (pH 3, 5, 9, and 11). The derivatives were most unstable at pH 11, forming crystals and orange colors. At pH 7, the half-life of five amino acid derivatives was 8−20 times the value for control red.

There is a report (*15*) that monascus derivatives of glutamic acid and amino acetic acid are more stable at pH 7.0 and 9.2 than at pH 3.0. Fabre (*18*) also reported that monascus extracts show a red color at acidic pH values and form crystals at an extreme alkaline pH. These results are similar to our data.

To analyze the color shift of pigments with pH, the absorption spectra for control red and the 5 derivatives were observed at different pH values. The spectrum of control red and the derivatives did not change with a changing pH, but the absorption intensity was varied (**Figure 4**). The highest absorption intensity was obtained at pH 7 for asparagine and threonine derivatives that have an uncharged polar group, at pH 5 for glycine and isoleucine derivatives that have a nonpolar aliphatic group, and at pH 3 for control red and the phenylalanine derivative that both have an aromatic ring. Changes in the absorption intensity of pigments with varying solution pH were affected by the R group of the amino acid. The highest and lowest values of the absorption intensity are known as hyperchromic and hypochromic shifts, respectively. The absorption spectrum of control red in a buffer solution at pH 3 was shifted toward the long wavelength. This bathochromic shift is probably related to the enhanced photostability of control red at pH 3.

Photostability of Pigments in Various Solvents. After 5 derivatives and control red dissolved in various solvents were exposed to sunlight, their half-lives were observed (**Figure 5**). Then, the values went through Tukey's test at $p \leq 0.05$. The stability of all the pigments decreased in descending order in hexane (a), ethanol (ab), propanol (ab), methanol (ab), ethyl ether (b), distilled water (c), chloroform (c), and acetonitrile (d). The symbols a−d indicate a statistically significant difference. Control red and all derivatives in various solvents showed a similar pattern of photostability, with the exception of control red in distilled water (**Figure 5**). Hexane was most favorable to the derivatives whereas acetonitrile and chloroform were the worst. On the other hand, the control red was most stable in water.

Good photostabilities of derivatives were observed not only in relatively hydrophilic solvents such as methanol, ethanol, and propanol but also in hydrophobic solvents such as hexane and ethyl ether. These results indicate that the photostability of pigments would be not related to the polarity of solvent. Overall, the stabilities of derivatives were at least 8 times greater than control red regardless of solvent type.

Stability of Pigments under UV Light. The photostability of pigments was tested under a UV light (365 nm wavelength), and the half-life was estimated (**Table 2**). The half-life of control red was 24.0 h while derivative half-lives were 168−351 h, corresponding to more than 7−15 times the control red value. All the pigments were much more stable under UV light than under sunlight, probably due to the low irradiation strength of UV light. However, the photostability pattern under UV irradiation was similar to that under sunlight. As with the results for sunlight, the derivatives were more stable under UV light than control red, with values similar to cochineal. On the other hand, the k values of the derivatives were 0.002−0.004 h^{-1}, which corresponds to less than one-seventh of the control red.

Effect of Storage Temperature on Pigment Stability. The temperature stability of control red and the five amino acid derivatives was tested. The pigments were incubated for 30 days at 4, 30, and 80 °C in darkness. The derivatives were fairly stable for 30 days at 4 °C with more than 95% of the original pigment (**Figure 6**). At 30 °C, more than 80% of the initial pigment amount remained, and at 80 °C 40−65% of the pigment remained. Approximately 62% of control red remained at 4 °C, and 29% remained at 30 °C after 30 days. At 80 °C, no control red pigment remained. The half-life of control red at 80 °C was 3.5 days. The derivatives are much more stable over a temperature range than control red. The 80−95% of derivatives remaining after 30 days of storage at both 4 and 30 °C indicates that they are not seriously degraded at room temperature in darkness.

Color Change of Pigments under Sunlight Irradiation. Pigment color change after an extended exposure to sunlight was visually evaluated. Control red gradually changed to a brown color while all the amino acid derivatives became a weaker red. The results indicate that there is a difference in the degradation patterns of control red and the derivatives.

Control red and the phenylalanine derivative were selected for further analysis. Photostability values were analyzed using a colorimeter (CIELAB colorimetric system). With an increasing exposure time, the L^* (lightness) and a^* (redness) values of both pigments gradually decreased (**Table 3**). However, the b^* value of control red was little changed whereas the b^* value of

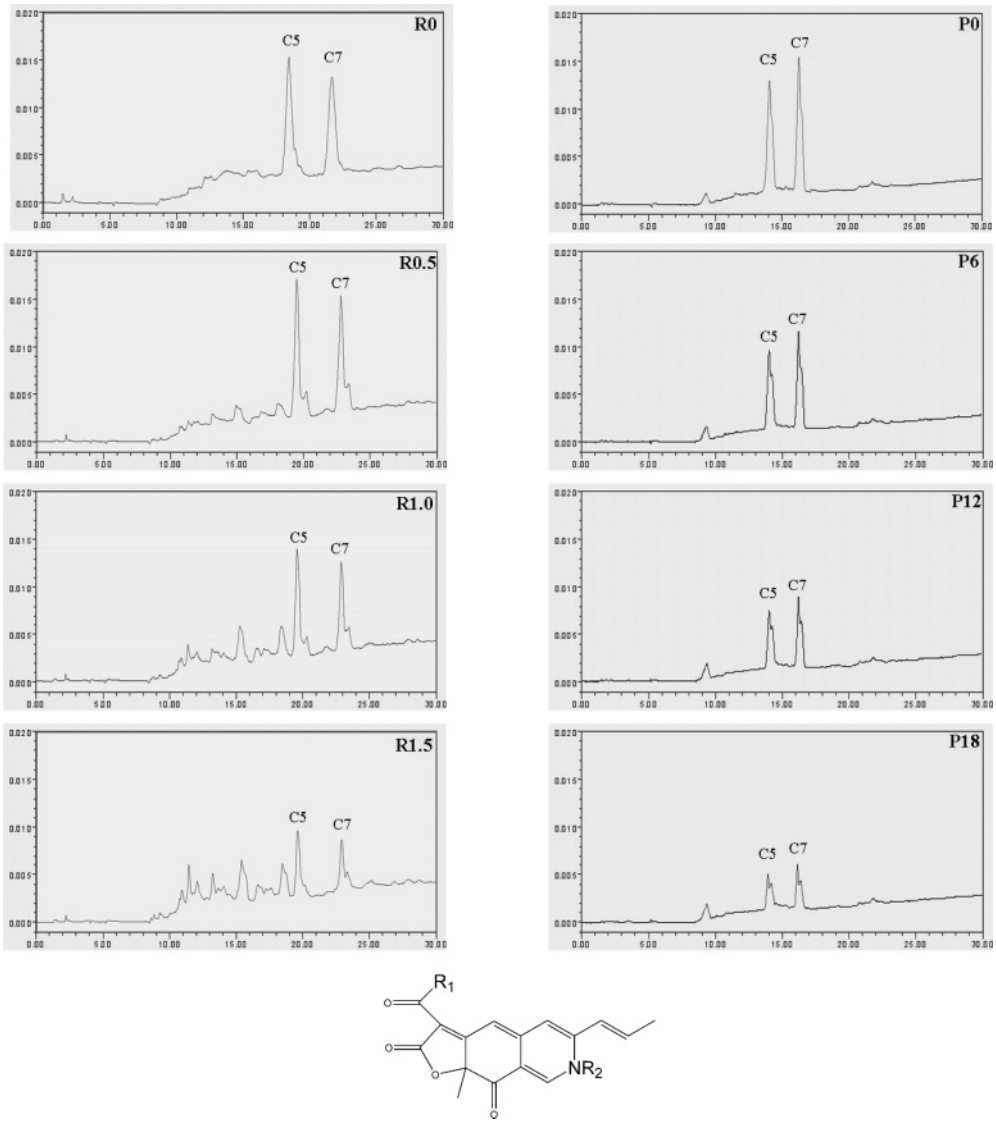

Figure 8. Degradation pattern of control red and the phenylalanine derivative after incubation. R = control red: R0, 0 h; R0.5, 0.5 h; R1, 1 h; R1.5, 1.5 h. P = phenylalanine derivative: P0, 0 h; P6, 6 h; P12, 12 h; P18, 18 h. C5 = C_5H_{11} on R_1, and C7 = C_7H_{15} on R_1. The average intensity of sunlight irradiation/h during the experimental period was 2.33 ± 0.26 MJ/m^2.

Table 3. Colorimetric Values for the Decoloration of Control Red and Phenylalanine Derivative under Sunlight Irradiation[a]

	control red exposure times (h)				phenylalanine derivative exposure times (h)			
param	0	0.5	1.0	1.5	0	6	12	18
*L**	61.50 ± 0.03	67.03 ± 0.08	75.06 ± 0.02	86.08 ± 0.01	56.65 ± 0.07	62.57 ± 0.10	68.91 ± 0.03	78.31 ± 0.02
*a**	49.38 ± 0.10	40.35 ± 0.20	26.99 ± 0.06	11.59 ± 0.03	60.40 ± 0.16	54.95 ± 0.27	46.44 ± 0.08	32.27 ± 0.05
*b**	47.29 ± 0.06	46.68 ± 0.14	42.33 ± 0.05	27.25 ± 0.02	38.79 ± 0.09	21.97 ± 0.14	14.72 ± 0.03	8.08 ± 0.03
chroma	68.37	61.70	50.20	29.61	71.78	67.26	48.77	33.27
hue angle (deg)	43.76	49.16	57.48	66.96	32.71	35.22	17.59	14.06

[a] The average intensity of sunlight irradiation/h during the experimental period was 2.33 ± 0.26 MJ/m^2.

the phenylalanine derivative was greatly decreased. Chroma values and hue angles were calculated from the values of *L**, *a**, and *b**. The chroma values of both pigments decreased in a similar pattern, moving toward a weaker color. However, the hue angle of control red increased whereas that of the phenylalanine derivative decreased, indicating that control red was shifted toward the yellow and orange whereas the phenylalanine derivative was shifted toward red (**Figure 7**).

The degradation patterns of control red and the phenylalanine derivative over time were analyzed by HPLC (**Figure 8**). Many new compounds appeared in control red after light treatment. However, the heights of the major peaks for the phenylalanine derivative only decreased without the appearance of additional peaks. Apparently, control red is degraded to other compounds while the phenylalanine derivative disappears without formation of new chromophores. The brown colors formed from control red are probably due to decomposition or combinations of new compounds. However, the color of the phenylalanine derivative remained a weak red, indicating that there are differences in the degradation patterns of control red and the phenylalanine derivative. A more detailed analysis is required.

In conclusion, the photostability of amino acid derivatives was much greater than that of the control red, which is produced by *Monascus* species under no adding condition of amino acids.. The derivatives were also much more stable than control red under various conditions such as changes in pigment concentration, pH, solvent, and temperature. Differences in photostability of control red and derivatives are supposed to depend on the degradation pattern of pigment molecules.

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Received for review May 4, 2005. Revised manuscript received July 11, 2005. Accepted July 12, 2005.

JF0510283