Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: http://www.tandfonline.com/loi/tbbb20

Structure of Green Pigment Formed by the Reaction of Caffeic Acid Esters (or Chlorogenic acid) with a Primary Amino Compound

Goro YABUTA, Yukimichi KOIZUMI, Kazuko NAMIKI, Mitsuhiko HIDA & Mitsuo NAMIKI

To cite this article: Goro YABUTA, Yukimichi KOIZUMI, Kazuko NAMIKI, Mitsuhiko HIDA & Mitsuo NAMIKI (2001) Structure of Green Pigment Formed by the Reaction of Caffeic Acid Esters (or Chlorogenic acid) with a Primary Amino Compound, Bioscience, Biotechnology, and Biochemistry, 65:10, 2121-2130, DOI: 10.1271/bbb.65.2121

To link to this article: http://dx.doi.org/10.1271/bbb.65.2121

Biosci. Biotechnol. Biochem., **65** (10), 2121–2130, 2001

Structure of Green Pigment Formed by the Reaction of Caffeic Acid Esters (or Chlorogenic acid) with a Primary Amino Compound

Goro YABUTA, Yukimichi KOIZUMI, Kazuko NAMIKI,[1] Mitsuhiko HIDA,[2] and Mitsuo NAMIKI[3]

Department of Fermentation Science, Faculty of Applied Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan
[1]*Sugiyama Jogakuen University, (Current address: 2-175 Yashirodai, Meitou-ku, Nagoya 465-0092, Japan)*
[2]*Tokyo Metropolitan University, (Current address: 7-2-12 Minamikoiwa, Edogawa-ku, Tokyo 133-0056, Japan)*
[3]*Nagoya University, (Current address: 2-175 Yashirodai, Meitou-ku, Nagoya 465-0092, Japan)*

Received April 26, 2000; Accepted June 20, 2001

A marked greening observed in some foods such as sweet potato, burdock, and others during food processing was shown to be due to green pigment formation by the condensation reaction of two molecules of chlorogenic acid or caffeic acid ester with one molecule of a primary amino compound under aeration in alkaline solution. Reduction of the green pigment by ascorbic acid or $NaBH_4$ gave a yellow product, which readily turn green and then blue in air. The reduced and acetylated product of the green pigment was identified to be a novel trihydroxy benzacridine derivative, and the yellowish ethanol solution of this product immediately turned green upon addition of butyl amine or diluted alkali. Therefore, the green pigment was assumed to be an oxidized quinone type product of trihydroxy benzacridine. This identification of the structure was supported by the correspondence of the measured absorption spectra with those calculated by the molecular orbital method. A possible charge transfer complex between products of different oxidation steps in green solution was proposed.

Key words: green pigment; chlorogenic acid; caffeic acid ester; amino compound; benzacridine derivative

A marked greening is observed in some vegetables, such as sweet potato, burdock, and others during some types of food processing and cooking, *e.g.* bread making and deep frying. Figure 1 shows steamed bread containing small cubes of sweet potato stored a fairly long time, which turned bluish green after standing overnight. The greening is prominent in the case of damaged or infected sweet potato, and especially under in alkaline conditions caused by additional baking powder, suggesting that it was increased by the oxidation of a polyphenol component such as chlorogenic acid by induced polyphenol oxidase activity.[1)] This greening was also shown to be prominent in the presence of amino acids.[1,2)] Greening of burdock during deep frying to make tempura is also attributed to its polyphenol component, chlorogenic acid.[3)] Greening is sometimes observed during alkali extraction of protein from sunflower meal and shown to be due to its chlorogenic acid.[4,5)]

Chlorogenic acid occurs widely in fruit, leaves, and other tissues of dicotyledonous plants, such as coffee bean and potato. Browning and sometimes bluish greening caused by chlorogenic acid have long been noted, and its name originates in the fact that this component turned green when plant tissue was treated with ammonia.[6–8)]

This greening is very interesting because the color green is rare in natural food systems, except for chlorophyll and its derivatives. There were several studies on the formation conditions of this greening,[9–14)] but the chemical structure of the green pigment is not yet known.

This study was undertaken to discover the chemical properties and structure of this green pigment and related products.

Materials and Methods

Chemicals. Caffeic acid esters were prepared in the usual manner,[15)] with caffeic acid and ethanol or methanol. Unless otherwise stated, analytical grade reagents were used in this experiment. Buffer solution was prepared with 0.1 M $NaHCO_3$–0.05 M Na_2CO_3 (pH 9.5).

Greening reaction. The greening reaction was done as follows unless otherwise noted. The aqueous or water-ethanol solution of caffeic acid ester and amino compound (usually 10 or 20 mM each) was kept in $NaHCO_3/Na_2CO_3$ buffer at pH 9.5 under continuous

To whom correspondence should be addressed. Goro YABUTA, Tel: +81-3-5477-2542; FAX: +81-3-5477-2622; E-mail: yabta@nodai.ac.jp

air bubbling at 50°C for 2 h.

Chromatography. Wako-gel (C-300) was packed in a column (benzene-ethyl acetate) and silica gel 60 silanized for reverse phase column chromatography (ethanol-H_2O). Thin layer chromatography (TLC) was done by silica gel $60F_{254}$ (ether, benzene-ethyl acetate) and silica gel RP-$18F_{254}$ (ethanol-H_2O).

Spectroscopy. H-1 and C-13 NMR spectra were measured with a JEOL α-400 spectrometer. The sample was dissolved in $CDCl_3$ and/or DMSO-d6. The MS spectrometer was a JEOL AX-505HA (EI). The UV spectrometer was a Shimazu UV-1600.

Method of molecular orbital calculations. The Pariser-Parr-Pople (PPP) method with new γ[17] was used for the calculations of absorption spectra of the pigments. Some fundamental energy parameters were estimated by the conventional method.[16-20] These energy parameters are functions of Slater's effective nuclear charge, which is calculated by the core charge (Q_r) and atomic number ((N atom)$_r$) by equation (1).

$$z_r = 0.65(\text{N atom})_r - 1.0 + 0.35Q_r \quad (1)$$

The charge (Q_r) of core (r) can be calculated with the atomic number, the number of lone pairs (N_n) and the sum of electron densities (q_σ) of the atom in σ-bond by equation (2).

$$Q_r = (\text{N atom})_r - 2 - 2(N_n)_r - \Sigma q_\sigma \quad (2)$$

Assuming all values of q_σ of C–C and C–O bonds to be unity, the charge of the aromatic carbon and oxygen of free hydroxyl and carbonyl groups are taken to be 1, 2, and 1, respectively. The charge of oxygen of a hydroxy bonding hydroxy group, however, was assumed to be 1.54, which was adopted suitably in case of 1-hydroxyanthraquinone having an intramolecular hydrogen bond. The charge of nitrogen is given by equation (3).

$$Q_N = 5 - (2q_{N\text{-}Ar} + q_{N\text{-}R}) \quad (3)$$

The electron density on nitrogen ($q_{N\text{-}Ar}$) of a σ bond between nitrogen and aromatic carbon was assumed to be unity. The value of $q_{N\text{-}R}$ of a σ bond between nitrogen and an alkyl group may be in the range between unity and two, because of the donating inductive electronic effect of the alkyl group. Therefore, Q_N can be in the range from 1 to 2 and in the case of a butyl group $Q_N = 1.5$ was adopted. All calculation was done in the case of Z = H (referred; Table 3) for convenience, because the effect of the alkoxy carbonyl group on the spectra is considered to be very small.

Color representations of absorption spectra. To compare the observed color of the products with the calculated results, Munsell's hue was estimated by the wavelength and oscillator strength of spectra as follows:

(a) The weighted mean chromatic coordinates were calculated with wavelengths (λ_k) and oscillator strengths (f_k) in the visible region by equations (4.1) and (4.2), where x_k and y_k are the chromatic coordinates of the spectral color of wave length (λ_k). S_k is sum of the stimulated coefficients and T_k is the transmittance.[21]

$$x_{bar} = \Sigma[(1 - T_k)S_k x_k] / \Sigma[(1 - T_k)S_k] \quad (4.1)$$

$$y_{bar} = \Sigma[(1 - T_k)S_k y_k] / \Sigma[(1 - T_k)S_k] \quad (4.2)$$

If the maximum value of oscillator strength is denoted by f_{max}, T_k can be given by equation (5), where T_0 is the transmittance at the wavelength of the maximum oscillator strength (f_{max}).

$$T_k = \exp[(f_k / f_{max}) \ln(T_0)] \quad (5)$$

Since chromaticness may change with the concentration of a pigment in solution, the value of T_0 was taken over the range between 1 and 70%. In equations (4.1) and (4.2) summation is made over the calculated wavelengths in the visible region.

(b) The dominant wave length (λ_d) is complementary to the wavelength corresponding to the coordinates (x_{bar} and y_{bar}).

(c) The Munsell's hue of λ_d was roughly evaluated from its chromatic coordinates.[22]

Results and Discussion

Effects of reaction conditions on greening

Aeration of chlorogenic acid in alkali produce only browning, and greening developed with the addition of amino acids such as glycine and alanine. Thus the effects of various phenol compounds and amino compounds on the development of greening were examined.

Polyphenol

The greening produced by caffeic acid esters such as methyl, ethyl and *n*-butyl esters is almost the same as that occurring with chlorogenic acid, but isopropyl ester gave a somewhat bluish green. Caffeic acid amide also produced greening.[3] However free caffeic acid, ferulic acid, isoferulic acid, and *p*-coumaric acid and their esters gave no greening product. Dehydrocaffeic acid ester and protocatechuic acid ester also proved negative for greening. It was then demonstrated that an ortho phenol like caffeic acid with a double bond in the side chain and a carbonyl group in the carboxylic acid ester are necessary for the greening.

Amino compound

The greening developed only with a primary amino

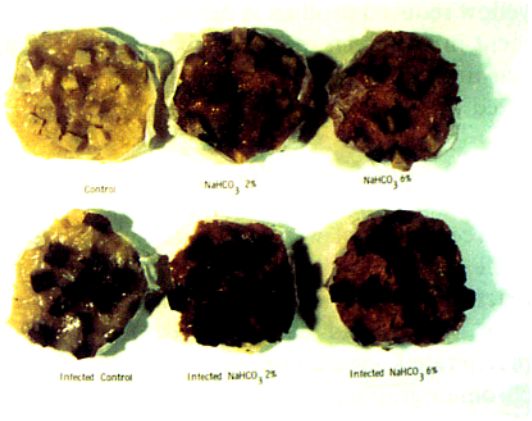


Fig. 1. Green Pigment Formation on Sweet Potato in Steamed Bread.

[Control]: With uninfected sweet potato. [$NaHCO_3$(2%)]: With uninfected sweet potato + 2% $NaHCO_3$. [$NaHCO_3$(6%)]: With uninfected sweet potato + 6% $NaHCO_3$. [Infected control]: With infected sweet potato. [Infected $NaHCO_3$(2%)]: With infected sweet potato + 2% $NaHCO_3$. [Infected $NaHCO_3$(6%)]: With infected sweet potato + 6% $NaHCO_3$.

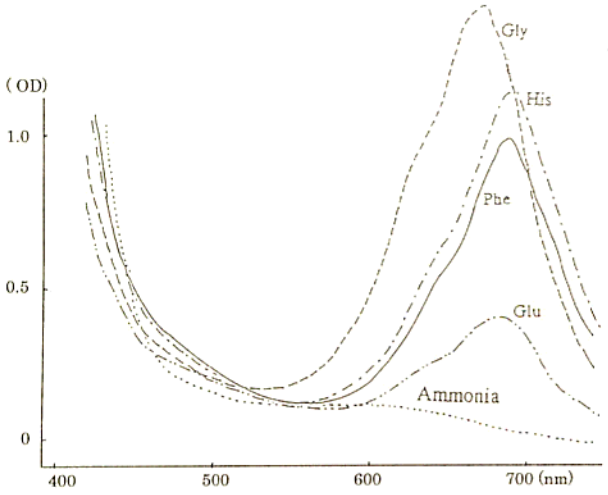


Fig. 2. Visible Absorption Spectra of Green Reaction Mixtures of Caffeate with Different Amino Compounds.

Ethyl caffeate + Amino acid (20 mM each), at pH 9.5, 50°C, 2 h.

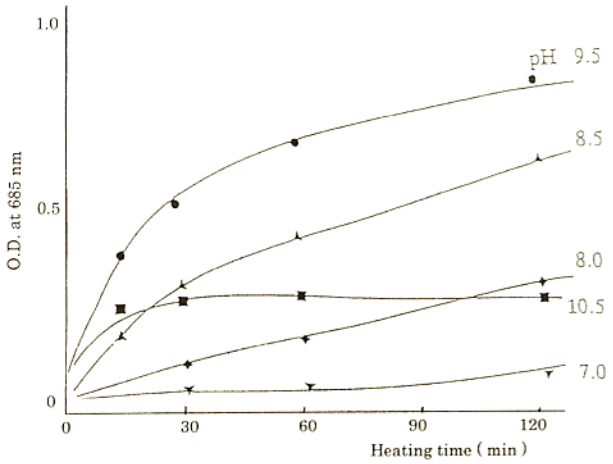


Fig. 3. Effects of pH on Green Color Formation.

The greening reaction with Et-caffeate and α-alanine (10 mM each, at 50°C) at different pH, determined at 685 nm.

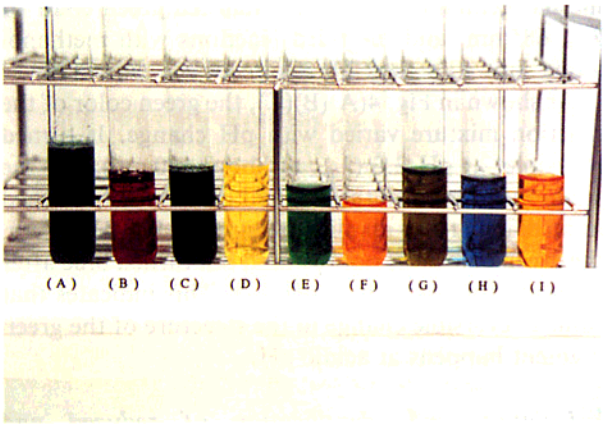


Fig. 4. Color Changes in the Green Pigment, Reduced Green Pigment, and Reduced and Acetylated Pigment by Changing pH and Reduction with Ascorbic Acid.

(A): Reaction mixture (Et-caffeate + Butylamine in buffer solution (pH 9.5) with aeration). (B): (A) + dil HCl (pH 1.0). (C): (B) + dil NaOH (pH 9.5). (D): Reduced and acetylated green pigment in EtOH. (E): (D) + butylamine. (F): Reduced green pigment. (G): (F) + alkali with aeration. (H): Stored overnight of (G). (I): (G) + Ascorbic acid.

group such as amino acid and alkyl amine, while secondary and tertiary amines and ammonia mostly showed only browning. The green color changed from clear green to deep green depending on the kinds of the amino compound as shown in Fig. 2, where the green color was intensified in the order Gly, Ala > His > Phe > > Glu. The λ_{max} (nm) of the green mixture were as follows; butylamine (673), Gly (678), Ala (682), and Phe (692). Serine and threonine, those α-amino acids containing a β-hydroxyl group, were negative for greening, though the reason is not known. Proline, a cyclic amino acid, and cysteine, an SH amino acid, were also negative.

Other reaction conditions for greening

As shown in Fig. 3, the greening occurred very slowly at pH 7.0, but in alkaline pH it developed immediately upon aeration, and increased with reaction time until saturation at about 2–3 h. The optimum pH of greening was 9.0–9.5. At higher pH as 10.5, the reaction occurred more rapidly, but did not increase in intensity and the resulting color was more bluish-green.

Some properties of green pigment

The green pigment developed from chlorogenic acid with an amino acid such as α-alanine was water soluble and not extracted with the usual hydrophobic

organic solvents. However, the pigment formed from isopropyl or higher alkyl caffeate with butylamine was extractable with some solvents, such as ethyl acetate or chloroform, though the green color was not as clearly green as when chlorogenic acid was used, but a pale blue-green.

The green pigment appeared to be a kind of amphoteric compound and precipitated at about pH 4.0 in the ethyl caffeate and butyl amine system. The precipitate was insoluble in ether but soluble in methanol. The green pigment precipitates obtained at pH 4 gave a bluish spot with tailing on silica gel TLC even upon rechromatography, and seemed to separate into blue and yellowish bands on silica gel column chromatography. In column chromatography with Sephadex XAD-7, the green reaction mixture of ethyl caffeate with alanine first gave brown eluates with 1% ammonia-water, so it probably contains oxidized phenolic products and unreacted materials. The second fractions with aqueous ammonia-methanol (8:2) gave a marked green color of λ_{max} 685 nm, and the third fractions with methanol alone showed a clear blue color of λ_{max} at 613 nm.

As shown in Fig. 4(A)(B)(C), the green color of the reaction mixture varied with pH change. It turned red violet at pH 2.0 (λ_{max} at about 520 nm); but the solution reverted to the original green when returned immediately to an alkaline pH around 9.0. However, the green color was not restored but turned blue after standing for over 2 h at pH 1-2. This indicates that some irreversible change in the structure of the green pigment happens at acidic pH.

Isolation and identification of reduced and acetylated green pigment

The green reaction mixture from ethyl caffeate with the butylamine system can be reduced to a yellow product by ascorbic acid or $NaBH_4$, and most of the reduced products can be extracted with ether. The reduced green reaction mixture gave several bands on the silica gel TLC with ether, and one yellow band (R_f 0.7, Fig. 4(F)) giving yellow fluorescence by UV lump (235 nm) readily turned green and then blue when TLC was standing in air. The color was reversed to yellow (Fig. 4(I)) *via* green by addition of ascorbic acid, as shown in Fig. 4(F)(G)(I). The λ_{max} of the absorption spectra of the colored solution are listed in Table 1. These facts indicate that the fluorescent yellow reduced product is derived from the green pigment in the green reaction mixture, but the product was difficult to isolate because it was very easily oxidized by air to the green pigment.

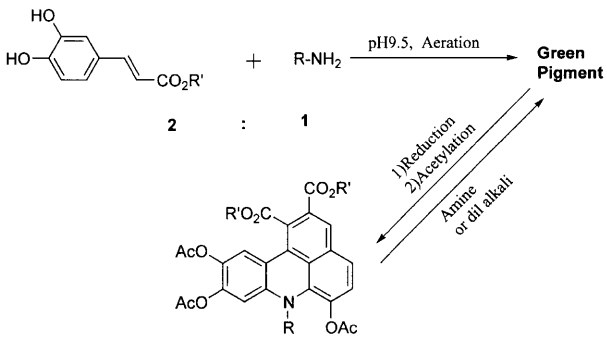
Table 1. Changes in Color and λ_{max} (nm) of the Reduced Green Pigment by Air Oxidation in Alkali and Reduction by Ascorbic Acid. (Refer to Fig. 4(F)(G)(I))

Material	λ_{max} (nm)	Color
(F) Reduced green pigment	441.0, 330.0, 288.0, 246.0	Yellow
(G) (F) + alkali (pH 9.5)	691.0, 332.0, 272.0	Green
(H) (G) after 24 h	639.0, 329.0	Blue
(I) (G) + Ascorbic acid	445.0, 330.0, 243.0	Yellow

Reduced and acetylated green pigment

The ether extracts of the reduced yellow solution of green pigment were acetylated using acetic anhydride and triethylamine at room temperature. The reaction mixture was washed with water, then dried over $MgSO_4$ and concentrated. After removal of precipitated crystalline products, the filtrate was chromatographed on a silica gel column with benzene-ethyl acetate (10:1 to 5:1). The crystalline products obtained in the earlier fractions gave white plates (mp. 77-78°C) from ethanol-water and were identified as ethyl caffeate diacetate from physicochemical data. The other crystalline product from the latter fractions gave yellow needles, mp. 188°C, by recrystallization with ethanol. The yellow needles obtained gave the following analytical data: Yellow needles, mp. 188°C, *m/z* at 591 (M^+, $C_{32}H_{33}O_{10}N$), 549 (M-42), 507 (M-84), 450 (M-142), 408 (M-183), NMR δ_H ($CDCl_3$, TMS) at 0.762 (3H, t, J=7.3, N-$CH_2CH_2CH_2$-CH_3), 1.145 (2H, m, N-CH_2CH_2-CH_2-CH_3), 1.342 (3H, t, J=7.0, C-1, O-CH_2CH_3), 1.414 (3H, t, J=7.0, C-2, O-CH_2CH_3), 1.557 (2H, m, J=7, N-CH_2-CH_2-CH_2CH_3), 2.27 (3H, s, C-10, $OCOCH_3$), 2.301 (3H, s, C-9, $OCOCH_3$), 2.378 (3H, s, C-6, $OCOCH_3$), 3.957 (1H, t, J=7.3, N-CH_2), 4.397 (2H, q, J=7.3, C-2, OCH_2CH_3), 4.401 (2H, q, J=7.3, C-1, OCH_2CH_3), 7.047 (1H, s, C-8, H), 7.200 (1H, d, J=8.6, C-5, H), 7.348 (1H, d, J=8.6, C-4, H), 7.468 (1H, s, C-11, H), 8.103 (1H, s, C-3, H).

The observed λ_{max} was 406, 323, and 245 nm. Compared with previous data[23] on the reduced and acetylated green pigment derived from methyl caffeate and butylamine, the structure was concluded to be diethyl-7-butyl-6,9,10-triacetoxybenz[k,l]acridine-1,2-dicarboxylate, as shown in Fig. 5.



Dialkyl-7-alkyl-6,9,10-triacetoxybenz[k,l]acridine-1,2-dicarboxylate

Fig. 5. Formation Reaction of Reduced and Acetylated Green Pigment from Caffeic Acid Ester and a Primary Amino Compound.

Greening of the reduced and acetylated product

The silica gel TLC of the reduced and acetylated crystalline products gave one spot having a characteristic yellow fluorescence with UV at 365 nm (Fig. 4(D)), and this spot turn green after standing overnight in air. The ethanol solution of the yellow crystalline product also turned clear green upon the addition of some amines, such as *n*-butyl amine and triethylamine, and diluted alkali such as dil. NaOH and $NaHCO_3$–Na_2CO_3 buffer (Fig. 4(E)). These facts indicate that the yellow crystalline product was easily deacetylated by amine or a weak alkali, and the phenolic product thus obtained was readily oxidized to give the green product. These results strongly support the idea that the green pigment is a novel benzacridine trihydroxy derivative. Except for some pyrolytic products,[23] very few benzacridine derivatives are known. The derivative in this study is the first one known to be formed from common plant components under ordinary conditions.

The blue pigment of the reaction mixture

As mentioned above, column chromatography of the green reaction mixture of Et-caffeate and α-alanine gave a blue fraction, showing absorption maximum at around 613 nm. The involvement of such a blue fraction, though minor, was also observed in other reaction systems. The blue color turned yellow in acidic solution, and it reverted to its original blue color when the pH was returned to alkaline after it was left standing overnight (Fig. 4(H)). The blue spot was also observed on TLC of the reduced yellow product mentioned above. The yellow spot of the reduced product turned green and then blue after standing in air or upon being sprayed with benzoquinone solution. The color was reversed *via* green to yellow upon spraying with ascorbic acid solution. Moreover, it was noted that when the green solution was acidified and left for several hours to give a yellow solution, and then returned to alkaline pH, the color changed to blue instead of the original green.

The blue products thus observed under different conditions are not necessarily the same compound; their chemical structures have not yet been identified. However, it is said that there exists a reversible oxidation-reduction relationship among yellow, green, and blue products of the identified benzacridine derivative.

Possible oxidation processes of the reduced product of green pigment

On the basis of those results, the chemical structures of the green pigment and other related products can be proposed to be oxidized structures of the identified benzacridine derivative of the reduced pigment product. The yellow reduced product has three phenolic hydroxy groups and may dissociate in alkaline solution, which will easily be oxidized stepwise by oxygen in air to give free radical and quinonoid products. Figure 6 shows the four-step successive oxidation processes of the reduced product to oxidation products, indicated by the most probable structures among several possible ones at each oxidation stage. The first step of one-electron oxidation will give free radicals such as Ia–Ic. These may readily be oxidized to give two-electron oxidized quinonoid products, IIa and IIb and/or their ionized products IIa′ and IIb′, which may be the green pigment (see following section). The product of a more oxidized stage, IIIa and/or IIIb, may correspond to the blue pigment.

Molecular orbital consideration of absorption spectra and colors of the green pigment and its related products

To investigate the correspondence of the probable structures of green pigment and related products to the observed color and absorption spectra, the molecular orbital calculation on the spectra, and colors of the probable structures of the pigments was made by a revised PPP (Pariser, Parr, and Pople) method[17,18] as described in the materials and method section.

The absorption spectra and color of blue or green pigment

The results of calculation of the absorption spectra, corresponding to the structures IIa, IIb, and IIIb and the dissociated structure of IIa′ and IIb′, are summarized in Table 3, in which only the wavelength over 380 nm is shown. The wavelengths over 780 nm are shown in parenthesis, since color is independent of light absorption in this wavelength region. The

Table 2. Changes in Color and λ_{max} (nm) of Reduced and Acetylated Green Pigment by Addition of Butylamine (in EtOH). (Refer to Fig. 4(D))

Time after addition	λ_{max} (nm)	Color
Red. Acetd. Comp. (D)	412.0, 330.0, 278.0, 237.0	Yellow
5 min	690.0, 442.0, 330.0, 246.0	Green
15 min	884.0, 690.0, 405.0, 330.0, 246.0	Greenish blue
1 h	884.0, 691.0, 483.0, 331.0, 249.0, 225.0	Greenish blue
24 h	884.0, 700.0, 642.0, 457.0, 393.0, 325.0	Blue
Addition of ASA*	460.0, 278.0, 237.0	Yellow

* ASA (ascorbic acid).

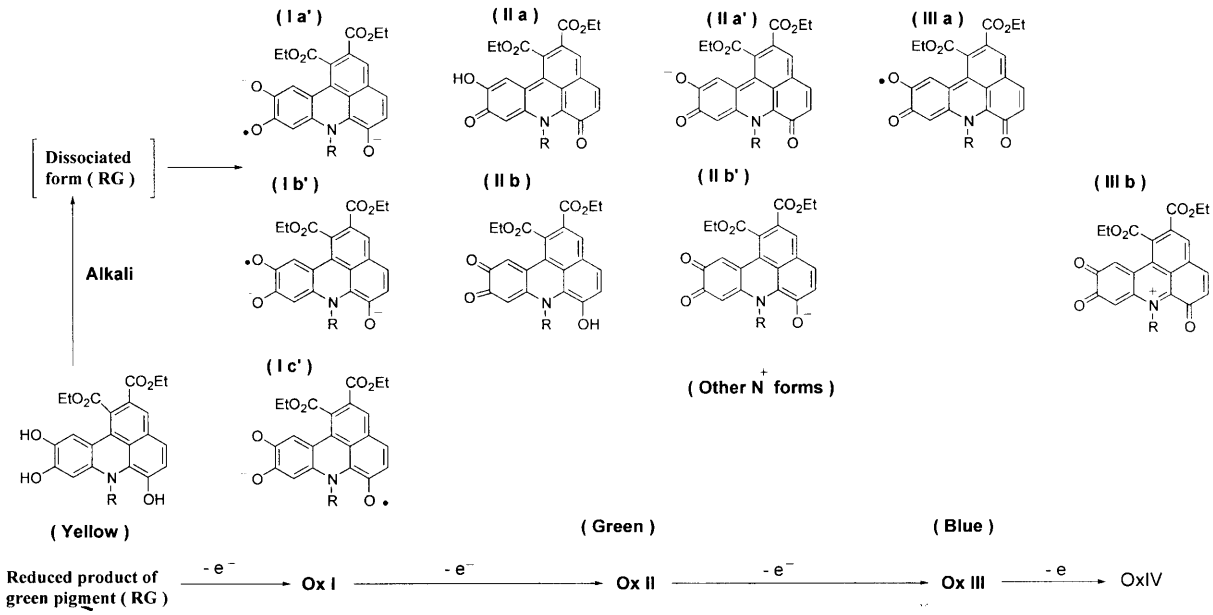


Fig. 6. Possible Oxidation Steps of the Reduced Product of the Green Pigment.

observed maximum wavelengths of acetylated RG ($Z=CO_2Et$, $R=Bu$, $R_1=COCH_3$) were 406, 323, and 245 nm while the calculated spectra of acetylated RG ($Z=H$, $R=Bu$, $R_1=COCH_3$) were 420 nm and 400.6 nm. Its calculated dominant wavelength is 565 to 570 nm, corresponding to 2.5GY to 10Y. The calculated result of the reduced product RG ($Z=H$, $R=Bu$, $R_1=H$), not yet isolated, is 397.7 nm/0.314, and its color will be 2.5GY to 10Y. As shown in Table 3, all structures of the oxidation products are expected to be colored blue or bluish green.

The structures, IIa′ and IIb′, can be presumably differentiated, because the terminal oxygen of these structures will not be equivalent to each other. Both structures may have absorption at a longer wavelength. The structure IIa′, however, can more suitably correspond to the blue or bluish green because the oscillator strength of IIb′ is quite small. The structure IIa′, however, can more suitably correspond to the deep blue or blueish green, while the calculated oscillator strength of IIb′ is quite small, and its color was expected to change from purple to blue with the concentration in solution. The green color of pigment changes to reddish purple or purple under acidic conditions. The calculated result suggests that the structure protonated at nitrogen was reasonably assigned to IIb^+, but not to IIa^+.

Contrary to the above speculation on the structure of colored products (Fig. 6), the calculated results show that the product (IIIb) may absorb at a longer wavelength than the former oxidation stage products (IIa) and (IIb′), and the color of the former was expected to be green, but the color of the the product (IIIb) may be blue or bluish green. However, it is noticed that in the theoretical calculations a clear distinction among blue, bluish green and a part of the green colors seemed difficult due to the error including in the molecular orbital calculations and estimation of the dominant wavelength. Also, the Munsell's hue will be rather crudely estimated.

Effects of alkyl group on absorption spectra of the green pigment

The maximum absorption wavelengths (λ_{max} (nm)) having different alkyl groups are arranged in increasing order as follows:

$$Bu(673) < -O_2CCH_2(678) < -O_2C(CH_3)CH(682) < phCH_2(-O_2C)CH(692).$$

Compared the inductive substituent constant ($\sigma_I = 0.05$) of carboxylate group with that of methyl group ($\sigma_I = 0.00$), the carboxylate group will some smaller electron donation than methyl group. Therefore the electron donations of alkyl groups are arranged in thefollowing order, the reverse of the above:

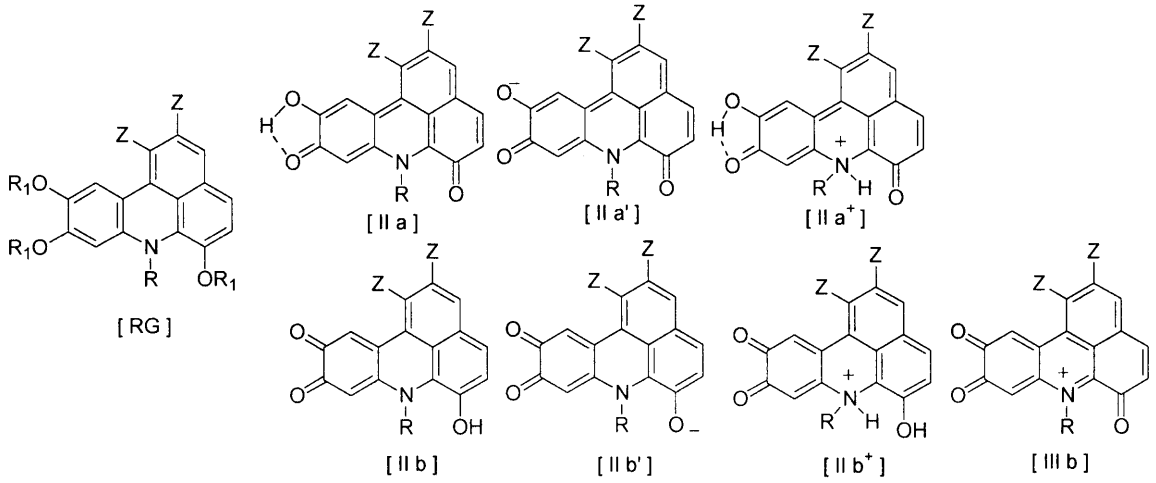
$$Bu > -O_2CCH_2 > -O_2C(CH_3)CH > phCH_2(-O_2C)CH.$$

Then the shift of the absorption may be concluded to be caused by the electron donating character of the alkyl groups. Calculation was done for different electron density (q_{N-R}) on nitrogen in the *N*-alkyl sigma bond, assuming that q_{N-Ar} is unity. The electron density (q_{N-R}) increases with an increase in the electron donation of the alkyl group. Table 4 shows that an increase in q_{N-R} sifts the calculated wavelength of the first absorption band IIa′ to a shorter wavelength.

Possibility of quinhydrone type charge transfer complex

The green pigment sometimes separated into blue

Table 3. Calculated Absorption Spectra and Color for Possible Structures of the Green Pigment and Related Products. (Calculated by PPP method, Refer to Fig. 6)



Chart

	λ_{max} (nm)/oscillator strength	Dominant wavelength nm/ Munsell's hue*
RG(Z=H, R=Bu, R_1=$COCH_3$)		
	420.0/0.136, 400.6/0.247	565-570/2.5GY-10Y
RG(Z=H, R=Bu, R_1=H)		
	397.7/0.314, (361.7/0.251)	565-570/2.5GY-10Y
IIa(Z=H)		
	(976.3/0.437), 629.0/0.174, 587.8/0.725, 497.0/0.502, 451.7/0.086, 419.7/0.069, 382.5/0.150	490-495/10BG-5BG
IIa'(Z=H)		
	(966.2/0.436), 669.9/0.462, 586.9/0.627, 509.2/0.307, 453.3/0.053, 421.0/0.074	480-485/7.5B-2.5B
IIb(Z=H)		
	776.4/0.458, 614.7/0.224, 434.5/0.019, 383.7/0.060	490-495/10BG-5BG
IIb'(Z=H)		
	(857.6/0.581), 628.8/0.166, 509.9/0.075, 424.2/0.011	560C/5.0P**
	382.6/0.044	435-485/10PB-7.5B
IIIb(Z=H)		
	755.4/0.120, 666.4/0.102, 583.5/0.248, 481.8/0.120, 414.5/0.163, 387.9/0.284	505-535/5G-2.5G
IIa^+(Z=H)		
	732.3/0.176, 711.4/0.876, 600.7/0.663, 501.4/0.144, 424.0/0.236	510-535/2.5G
IIb^+(Z=H)		
	731.1/0.166, 507.6/0.136, 464.3/0.016	805-835/2.5RP-10P

* Munsell's hue; GY (greenish yellow), Y (yellow), BG (bluish green), B (blue), G (green), RP (red purple), P (purple), PB (purple blue).
** Transmittance (T_0): upper; 1%, bottom; 70%: 560C means the complemental color of 560 nm.
The wavelengths in parentheses were independent of the color.

Table 4. The Effects of *N*-alkyl Group in the Benzacridine (IIa') (in Fig. 6) on the Calculated Absorption Spectrum. (Calculated by PPP Method[17])

	λ_{calc}/nm
q_{N-R}	IIb'
1.5	(966.2), 669.9, 586.9, 509.2, 453.3, 421.0
1.4	(922.4), 675.7, 594.3, 506.0, 447.9, 419.4
1.3	(882.7), 682.0, 602.7, 503.5, 442.9, 418.8
1.2	(848.2), 688.2, 611.0, 500.7, 438.7, 418.6
1.1	(817.0), 694.8, 619.6, 498.9, 435.1, 418.7

* The wavelengths in parentheses were independent of the color.

and yellow bands on TLC and column chromatography, suggesting some possibility of a bluish green or green charge transfer complex. Then the possibility of formation of a charge transfer complex was discussed by the molecular orbital method. The pair of hydroquinone and *p*-benzoquinone is well known to form quinhydrone, a marked bathochromic charge transfer complex.

The formation reaction of the charge transfer complex profitably proceeds when the difference between the ionic potential of the electron donor (D) and the electron affinity of the electron acceptor (A) is small enough. Since the ionization potential and the electron affinity equal the values of opposite sign of homo and lumo energies, respectively, the formation of a charge transfer complex may be possible when the value of $\Delta\varepsilon_{CT}=\varepsilon(A)_{lumo}-\varepsilon(D)_{homo}$ is sufficiently small. The pairs listed in Table 5 are expected to form

charge transfer complexes, comparing $\Delta\varepsilon_{CT}$ of the pairs with that of the hydroquinone and *p*-benzoquinone pair. The results indicated that the pair consist of highly oxidized product [IIIb] as the electron acceptor and the reduced or less oxidized products, such as RG or IIa, IIa′ and IIb′, as the electron donors; and suggest that these pair formation resulted in the fairly stable green or bluish green color of the reaction mixture.

Proposed mechanism of green pigment formation from Et-caffeate and amino compound

The formation mechanism of the trihydroxy benzacridine is not yet clear, but there exist various possible pathways. Among them, Fig. 7 shows one pathway where the orthoquinoid product of caffeate react first with an amino compound to give a schiff base product and condensation with another quinoid product followed by intermolecular Diels-Alder type cyclization to give a benzacridine structure.

Table 5. Possible Quinhydrone Type Charge Transfer Complexes. (Calculated by PPP Method[17])

Ionic potentials of the electron donors $-\varepsilon(D)_{homo}$/eV	The electron affinities of the electron acceptors $-\varepsilon(A)_{lumo}$/eV	$\Delta_{\varepsilon CT}$/eV
hydroquinone [HQ]	*p*-benzoquinone [Q]	
7.49	5.40	2.09
[RG]	[IIIb]	
7.54	5.47	2.07
[IIa]	[IIIb]	
6.87	5.47	1.40
[RG]	[IIa]	
7.54	5.31	2.23
[IIa′]	[IIIb]	
6.78	5.47	1.31
[IIb′]	[IIIb]	
7.52	5.47	2.05

$D + A \rightleftarrows D^+A^-$; $\Delta_{\varepsilon CT} = [\varepsilon(A)_{lumo} - \varepsilon(D)_{homo}]$.
Structure of each [ ] symbol: see Table 3 in chart.

Conclusion

Elucidation of chemical structure and formation mechanism of the green pigment observed in sweet potato and other plant food materials has long been noted as a pending problem. In this study it was demonstrated that the green pigment is an oxidized form of a newly discovered benzacridine trihydroxy derivative formed by the condensation reaction of two molecules of oxidized chlorogenic acid or caffeic acid ester with one molecule of a primary amino compound under aeration in an alkaline condition.

This is very interesting from the viewpoint that such a clear green color as that shown in the photo is rare in food systems, except for chlorophyll, and moreover because the benzacridine compound is rare in natural products. This green color is fairly stable in the reaction mixture, but there are still some problems in isolation and purification, so the possibility of its use as a kind of food colorant remains to be examined, though the green pigment showed no mutagenicity in the Ames test.

The structure of caffeate involving ortho-phenol, a double bond, and carbonyl in the side chain is favorable for easy oxidation in alkaline conditions by oxygen from air, to give semiquinone and ortho quinone products. Such reactive caffeate derivatives

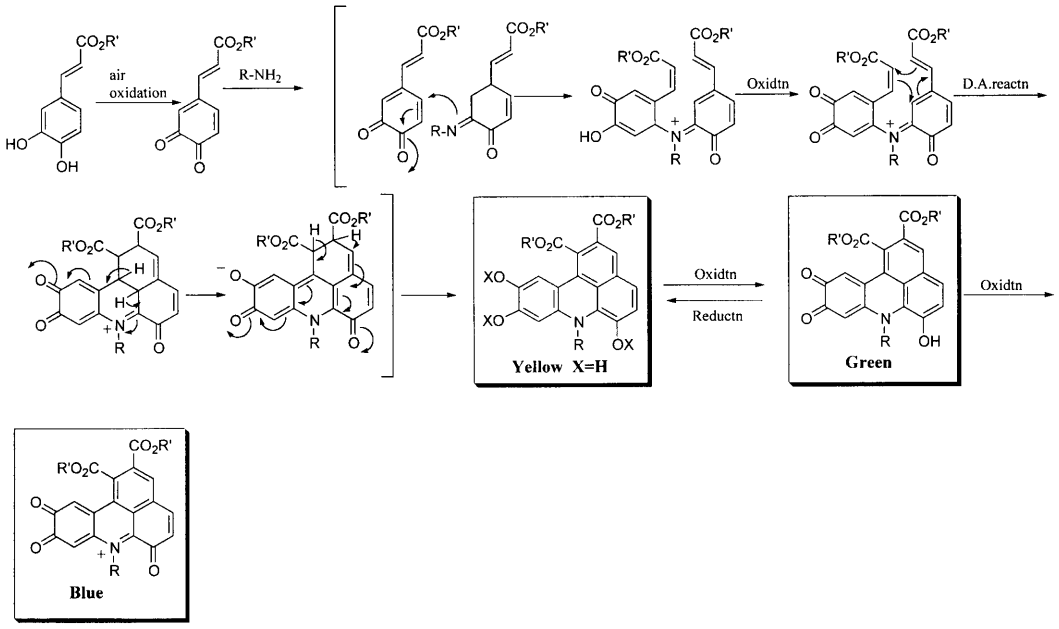


Fig. 7. Proposed Mechanism of Green Pigment Formation from Et-caffeate and Amino Compound.

alone can lead to the formation of polymerized brown products. Addition of an amino compound, however, brought about a significant changes in the reaction system, i.e. the combination of a nucleophilic primary amino compound with the oxidized caffeate molecules resulted in the formation of more stable heterocyclic compound, a benzacridine trihydroxy derivative. Involvement of nitrogen in the ring structure causes a bathocromic effect to give green color due to formation of a merocyanine structure, and an alkyl group on nitrogen may increase the electron-donating activity of the nitrogen atom.

In this study, the molecular orbital calculation on absorption spectra and colors of the probable structures of oxidized trihydroxy benzacridine provided very important information, which reasonably indicate that the two and three step oxidized structures have the estimated absorption spectra and colors corresponding to those of observed green and blue pigments.

The results of the molecular orbital calculation further indicated enough possibility to form quinhydrone type charge transfer complexes between several pairs of different oxidation stage products of trihydroxy benzacridine to give the green pigment. Then, it is concluded that the green and blue-green pigments observed in sweet potato and other plant food materials are quinone or semiquinone type products of trihydroxy benzacridine derivatives or charge transfer complexes of the quinhydrone type between them.

Acknowledgments

We are grateful to Drs. T. Hayashi, M. Isobe, T. Kondo, and I. Uritani of Nagoya University for their useful advice and help.

References

1) Uritani, I. and Muramatsu, K., Studies on the phytopathological chemistry of black-rottin potato. Part 2. Some factors concerning the color changes of the injured part. *Nippon Nogei Kagaku Kaishi* (in Japanese), **27**, 781–785 (1953).
2) Uritani, I., Hoshiya, I., and Takita, S., Phytopathological chemistry of black-rotten sweet potato. Part 11. The mechanism for greening occurred on the sound port next to the injured, when dipped in sodium bicarbonate solution (2). *Nippon Nogei Kagaku Kaishi* (in Japanese), **27**, 785–789 (1953).
3) Mano, Y., Kuratomi, M., Ouchi, K., and Takiguchi, M., The greening reaction of "Gobo" (Arctium Lappa L) Part 1. Separation and identification of chlorogenic acid. *Eiyo to Shokuryo*, **16**, 78–81 (1963).
4) Sabir, M. A. and Sosulski, F. W., Finlayson, A., Chlorogenic acid-protein interactions in sunflower. *J. Agric. Food Chem.*, **22**, 575–578 (1974).
5) Nakabayashi, T., Preparation of coffee-like brown pigments from the extract of common sunflower seed. *J. Food Sci. Technol. Jpn.* (in Japanese), **31**, 4508 (1984).
6) Payen, Untersuchung des Kaffees. *Ann.*, **60**, 286–294 (1846).
7) Gorter, K., Beitrage zur Kenntniss des Kaffees. *Ann.*, **358**, 327–348 (1908).
8) Politir, J., Sur une nouvelle methode concernant la localisation microchemique de l'acid chlorogenique et destanins dans les plantes. *Compt. Rend.*, **225**, 954–956 (1947).
9) Horikawa, H., Okayasu, M., Wada, A., and Kusama, M., Studies on the green pigments formed from chlorogenic acid and amino acid. Part 1. Isolation and purification of green pigments on Sephadex column chromatography. *J. Food Sci. Technol. Jpn.* (in Japanese), **18**, 115–118 (1971).
10) Nakatani, C. and Karasawa, I., Spectrophotometric studies on the behavior of chlorogenic acid in alkaline aqueous solutions. *Jpn. Soc. Food Nur.* (in Japanese), **32**, 47–53 (1979).
11) Nakatani, C. and Karasawa, I., Separation of the pigments produced as a result of greening of chlorogenic acid. *J. Jpn. Soc. Food Nur.* (in Japanese), **32**, 163–167 (1979).
12) Matsui, T., Greening pigments produced by the reaction of ethyl caffeate with methylamine. *J. Nutr. Sci. Vitaminol.*, **27**, 573–582 (1981).
13) Horikawa, H. and Furuya, E., Color reaction of amino acid and proteins with ethyl caffeate. *J. Jpn. Soc. Food Nur.* (in Japanese), **41**, 299–303 (1988).
14) Watanabe, S., Ushizawa, Y., and Kusama, M., Effect of pH on green pigments produced from chlorogenic acid and glycine. *J. Jpn. Soc. Food Sci. Tech.* (in Japanese), **43**, 1–6 (1996).
15) Carboxylic Esters. In "Synthetic Organic Chemistry", eds. Wagner, B. R. and Zook, D. H., John Wiley & Sons, New York, pp. 479–532 (1953).
16) Hida, M., A perturbational molecular orbital method for the absorption spectra of aromatic compounds. *Dyes and Pigments.*, **28**, 217–232 (1995).
17) Nishimoto, K., A MO theoretical study of organic dyes I. Effect of chemical softness on the electronic spectra. *Bull. Chem. Soc. Jpn.*, **66**, 1876–1880 (1993).
18) Hida, M., Symposium of Dyeing Chemistry, **39**, 57 (1999).
19) Titz, M. and Nepreas, M., Electronic structure and properties of polynuclea aromatic ketones and quinones I. Electronic spectra and π-electronic structure of some polynuclear quinones. Collection Czechoslov. *Chem. Commun.*, **37**, 2674–92 (1972).
20) Brown, R. D. and Heffernan, M. L., Study of formaldehyde by a 'Self-consistent electro negativity' molecular-orbital method. *Trans. Faraday Soc.*, **54**, 757–64 (1958).
21) Hida, M., Shikisai Kagaku (in Japanese), Maruzen 71 (1998).
22) Nihon Shikisai Gakkai, Shikisai Kagaku Handbook, Tokyo Daigaku Shuppankai 1352 (1980).
23) Yabuta, G., Koizumi, Y., Namiki, K., Kawai, T., Hayashi, T., and Namiki, M., Novel benzacridine derivative in the green pigment from methyl caffeate and butylamine. *Biosci. Biotechnol. Biochem.*, **60**, 1701–1702 (1996).

24) De Jongh, D. C. and Evenson, G. N., The pyrolysis of 2H-naphth[1,8-c,d]isothiazole-1,1-dioxide and its 2-phenyl analog. *J. Org. Chem.*, **37**, 2152–2154 (1972).
25) Namiki, M., Yabuta, G., Yano, M., and Koizumi, Y., Development of free radicals during the greening reaction of caffeic acid ester (or chlorogenic acid) and a primary amino compound. *Biosci. Biotechnol. Biochem.*, **65** (10) 2131–2136 (2001).