A New Red Coloration Induced by the Reaction of Oxidized Lipids with Amino Acids

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The characteristics of the red coloring reaction of oxidized lipids were studied using a stereoisomer (R1) of 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal and amino acids. Maximum color formation was observed at neutral pH and with a higher concentration of amino acid. This red coloration was observed with almost all α-amino acids tested, β-Ala and peptides of Gly but not with amides, amines, ammonia, cytosine, and the methyl ester of Gly. Each amino acid yielded its own red pigments: the red pigments were eluted at distinct positions with reversed-phase HPLC and showed slightly different absorption maxima (510−524 nm). Fluorescence on the red pigments was nil. The skeletons of ^{14}C- and ^{3}H-labeled amino acids were incorporated into the red pigments, and both carbonyl groups (IR ν 1740, 1690 cm^{-1}) of R1 were responsible for formation of the red pigments. These reaction characteristics were different from those of reducing sugars, ascorbic acid, and related substances.

Keywords: *Red coloration; oxidized lipid; amino acid; red pigment; reaction characteristics*

INTRODUCTION

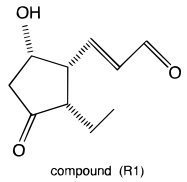
Brown discoloration in foods and biological systems is largely induced by aminocarbonyl condensation. This reaction leads to desirable and undesirable changes in raw, dried, cooked, and/or stored foods. In biological systems, "lipofuscin" or "ceroid", age pigments that are brownish, polymerized pigments are formed during aging processes (Porta and Hartrott, 1969; Yin, 1996). A great number of intermediate and highly reactive low molecular weight compounds such as carbonyls have been identified; however, the chemistry of colored materials, final products of the Maillard reaction (melanoidins), has remained unknown (Namiki, 1988; Friedman, 1996; Cohen and Ziyadeh, 1996).

As for red discoloration in foods, there are few reports on brewed products such as soy sauce, miso, and sake and on stored dried foods or vegetables. The former was induced largely by Amadori products formed by the reaction of reducing sugars with amino compounds during the aging processes (Hashiba et al., 1981; Hashiba, 1986). In the latter cases, it was demonstrated that dehydroascorbic acid, an oxidized form of ascorbic acid, reacted with amino acids to form red pigment via an intermediate aminoreductone (Koppanyi et al., 1945; Ranganna and Setty, 1968, 1974a,b; Hayashi et al., 1985).

Most of the coloration described above is based on the reaction of hydrophilic carbonyls such as a reducing sugars, ascorbic acid, and their related substances. We have studied lipophilic red pigment-forming substances (RPSs) formed during autoxidation of polyunsaturated fatty acids and their esters with three or more double bonds. The RPSs have a potent red coloring potential when reacted with amino acid (Nakamura, 1985) and have a common structure that is a conjugated carbonyl with a hydroxycyclopentanone ring. In previous papers, chemical structures of 10 types of RPSs were identified (Nakamura and Hama, 1988; Hama et al., 1992) and maximum production of the RPSs were determined quantitatively (Hama et al., 1990). In the present work, the reaction characteristics of RPSs with amino acids for the formation of red pigments was investigated using an RPS, 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal, which is the most abundant RPS obtained by oxidation of $n - 3$ fatty acids and/or their esters.

MATERIALS AND METHODS

Reagents. A stereoisomer of 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal, corresponding to R1 in our previous paper (Nakamura, 1985), was prepared from autoxidized methyl linolenate by the same method as described (at 40 °C, for 3 days). Reagents used in this study were of special grade



compound (R1)

unless otherwise specified. Glycine (Gly, Wako Pure Chemical Ind. Jpn) was recrystallized before use. L-Alanine (Ala), L-valine (Val), L-leucine (Leu), L-phenylalanine (Phe), L-cystine (Cys), L-cysteine (CysSH), L-proline (Pro), L-hydroxyproline (Hyp), β-alanine (β-Ala), methyl ester of Gly (HCl salt), methylamine (first grade), hexylamine (first grade), forma-

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mide, cytosine (first grade), and ammonia solution were purchased from Wako Pure Chemical Ind. Glycylglycine $(Gly)_2$ and glycylglycylglycine $(Gly)_3$ were products of Sigma Chemical Co. [2-^{14}C]Gly (3.7 MBq/mL) and [3,4-^{3}H]Val (37 MBq/mL) were from DuPon NEN (U.K.). Solvents used for HPLC analysis were HPLC grade of Wako Pure Chemical Ind.

Coloration of RPS with Nitrogenous Compounds. R1 (10 μg) was dissolved in 0.5 mL of MeOH and reacted with 1.0 mL of aqueous solution of nitrogenous compounds such as amino acids, esterified amino acid, peptides, imino acids, amines, amide, pyrimidine base, and ammonia. The mixture was incubated with shaking, and the color development of the each mixture was observed using a Hitachi 200-10 spectrophotometer (Hitachi Ltd., Tokyo, Jpn).

High-Performance Liquid Chromatography (HPLC). The red mixture was applied to reversed-phase HPLC on μ-Bondapak C18 (3.9 mm × 30 cm, Waters Associate Inc., Milford, MA) and eluted with 50% MeOH at a flow rate of 0.5 mL/min. Two UV−vis detectors (L-4200 and L-4250, Hitachi Ltd.) and a fluorescent detector (JASCO 821-FP, Japan Spectroscopic Co., Tokyo, Jpn) were connected in series to the column to monitor the reaction. Namely, absorption at 515 and 230 nm and a fluorescent intensity (excitation at 370 nm, emission at 450 nm) were detected simultaneously. UV−vis spectra of the red pigments were also recorded using a UV−vis detector (L-4250, Hitachi Ltd.).

Incorporation of Amino Acids. Incorporation of amino acids to the red pigments was examined using isotope-labeled amino acids. R1 (50 μg) in 25 μL of MeOH was incubated with 50 μL of [2-^{14}C]Gly (3.8×10^4 dpm) or 50 μL of [3,4-^{3}H]Val (2.0×10^4 dpm) in 0.5 M cold amino acid, respectively. Unreacted amino acid was removed by HPLC on TSK gel ODS-120T (4.6 × 250 mm, Tosoh, Jpn) eluted with 50% MeOH at a flow rate of 0.5 mL/min. The fractions containing the red pigments were recovered and reanalyzed by HPLC under the same conditions as described above. Elution of the red pigments was monitored by absorption at 515 nm. Fractions of 0.5 mL were collected through the entire run, and then 7 mL of Aqua Zol (DuPon NEN) was added. Radioactivity was measured using a liquid scintillation counter (2250 CA, Hewlett-Packard).

Preparation of the Red Pigment. After coloration with Gly (pH 7, 60 °C, 90 min), to the resultant deep red solution was added $CHCl_3$, followed by vigorous shaking and centrifugation, and the upper water layer containing the red pigment was applied to anion-exchange chromatography on a DEAE-Sephadex A-25 column (1.2 mL, Cl^- form, Pharmacia Fine Chem., Uppsala, Sweden). After removal of unreacted Gly by washing the column with distilled water, the adsorbed red pigment was eluted with 0.01 N HCl/MeOH. The Gly free methanolic eluate was concentrated to a small volume by rotary evaporator at iced-water temperature under the pressure below 25 mmHg, and the pigment was further purified by HPLC on a DELTAPAK C18 cartridge column (2.5 cm × 10 cm, Waters Associates, Inc.), which was eluted with 50% MeOH at 5.0 mL/min. The red pigment was pooled, and the solvent was removed in vacuo.

Infrared Spectroscopy. IR spectra of the red pigment and R1 were measured in a KBr disk and in $CHCl_3$, respectively, with an EPI-G21 Hitachi IR spectrophotometer (Hitachi Ltd.).

RESULTS

Coloration Characteristics of RPS. The colorless mixture of R1 and Gly (0.5 M, pH 7.0) changed gradually to simple red by incubation at 45 °C. The UV−vis spectral analysis of the colored mixtures indicated the presence of two major absorption maxima (Figure 1). One present at 515 nm derived from the red pigment, and the other present at 230 nm derived from conjugated carbonyl of the unreacted R1. Absorption of the former increased with incubation time in contrast to a decrease in the latter. No absorption other than red was observed in the visible region. The time course for

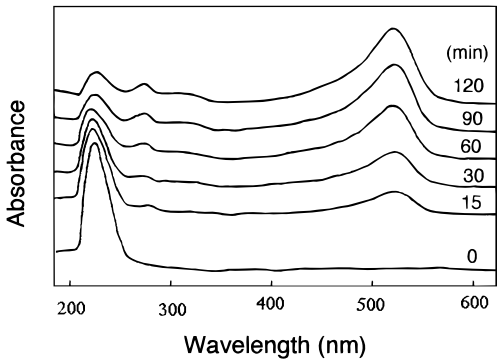

Figure 1. UV−vis spectra of the red mixtures derived from a stereoisomer of 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal (R1) and Gly. R1 (10 μg) in 0.5 mL of MeOH was added with 1.0 mL of 0.5 M Gly (pH 7.0) and incubated at 45 °C. The spectra were measured in 3 mL of 50% MeOH.

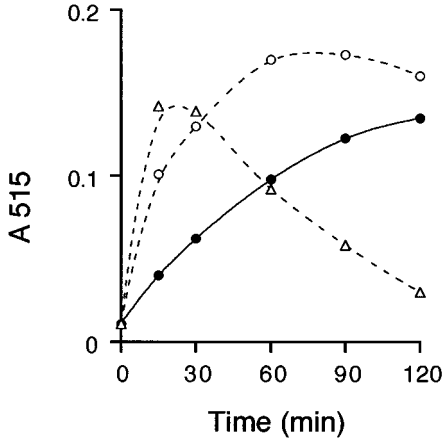

Figure 2. Time course of the formation of the red pigment. Reaction conditions other than temperature were the same as for Figure 1. Absorbance at 515 nm of the mixture was measured: −●−, incubated at 45 °C; −○−, incubated at 60 °C; −△−, incubated at 75 °C.

the formation of the red pigment at different temperatures was monitored by measuring the absorption of the colored mixture at 515 nm (Figure 2). In boiling water, the reaction mixture changed shades of red for several seconds and turned clear or very thin yellow. At 75 °C, red coloration occurred rapidly but faded in short time. On the other hand, gradual formation of the red pigment was noted at a lower temperature (45 °C). Since maximum formation of the pigment was observed when the mixture was incubated at 60 °C for 90 min, this condition was used for the following assays.

Effects of the concentration of Gly and pH of the reaction mixture on the red coloration were then investigated. As shown in Figure 3, a large excess of Gly and neutral pH of the reaction mixture was essential for higher red coloration. The mixture was not colored after incubation with Gly at pH 4. The reason may relate to inhibition of the aminocarbonyl reaction due to ionization of an amino group of Gly, in acidic solution. On the other hand, a brown coloration was observed in the case of reaction with an alkaline Gly solution. Interestingly, the red pigment formed at neutral pH turned brown when the colored mixture was alkalized, and this phenomenon was reversible.

Coloration of RPS with nitrogenous compounds other than Gly was examined and the results are listed in Table 1. β-Ala, peptides of Gly, and almost all α-amino acids, except for Cys and CysSH, gave a red coloration,

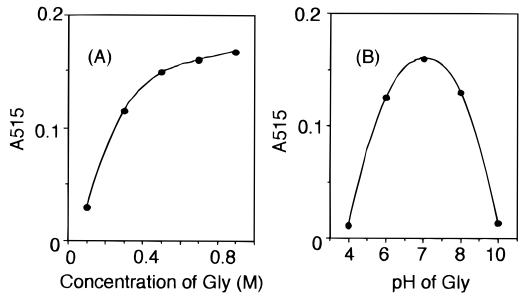

Figure 3. Effects of concentration and pH of Gly solution on red coloration. Various concentrations (A) and various pH (B) of Gly solution (1 mL) were reacted, respectively, at 60 °C for 90 min. Other conditions were the same as for Figure 1.

Table 1. Coloration of R1 with Nitrogenous Compounds

compd	color development	compd	color development
α-amino acid		imino acid	
Gly	red	Pro	golden yellow
Ala	red	Hyp	golden yellow
Val	red	amine	
Leu	red	methylamine	yellow
Lys	red	hexylamine	yellow
Phe	red	formamide	colorless
Cys[a]	colorless	cytocine[a]	colorless
CysSH[a]	colorless	ammonia	colorless
β-Ala	red		
methyl ester of Gly	colorless		
peptides of Gly			
$(Gly)_2$[a]	red		
$(Gly)_3$[a]	red		

[a] Saturated solution was used.

but not so the methyl ester of Gly. A characteristic golden yellow coloration was obtained with imino acids (Pro, Hyp). Yellow to brown coloration occurred when reacting with amines (λ_{max} 443 nm with hexylamine, λ_{max} 438 at methylamine). When these colored solutions were separated into lipophilic and hydrophilic phases with use of the solvent system ($CHCl_3$/MeOH/water, 8/4/3, v/v), these brownish pigments distributed into the lipophilic part (lower), whereas the red pigments derived from RPSs and amino acids went into the hydrophilic part (upper). On the other hand, no coloration was obtained with formamide, ammonia, and cytosine.

Structural Characteristics of Red Pigments. *Diversity of the Red Pigments.* The resultant colored mixture was separated by the reversed-phase HPLC. Each amino acid yielded its own red pigments: one red pigment peak was detected in cases of Gly and Ala, whereas two peaks were detected in those from Val, Leu, and Phe (Figure 4). The higher the molecular weight of the amino acid, the longer time the resultant pigments were retained in the column. UV−vis spectra of these red pigments are shown in Figure 5. Absorption maxima (λ) of these pigments were slightly different: Gly at 515, Ala at 518, Val at 522 (peak 1 in Figure 4), 520 (peak 2 in Figure 4), Leu at 522 (peak 1), 521 (peak 2), and Phe at 525 (peak 1), 524 nm (peak 2). As described in the previous section, red coloration was also observed with peptides of Gly. However, in terms of spectral analysis, the red pigments derived from the peptides differed and also differed from those derived from amino acids (Figure 5). Fluorescence characteristics of the red pigments were examined simultaneously by HPLC. Colored mixtures derived from R1-Gly showed one red pigment peak coeluted with a fluorescent peak (Figure 6A). On the other hand, one fluorescent peak and two red pigment peaks without fluorescence were detected in the chromatograms of R1-Val (Figure 6B) and also in R1-Leu and R1-Phe. The red pigment and fluorescent peaks from R1-Gly were eluted separately when small amounts of acetic acid was mixed with the eluent, although the peak area of the red pigment decreased significantly. Time course for the formation of the red pigment and fluorescent products derived from R1-Gly was also examined by HPLC. The fluorescent peak area was increased with incubation even when the red color began to decrease (Figure 7).

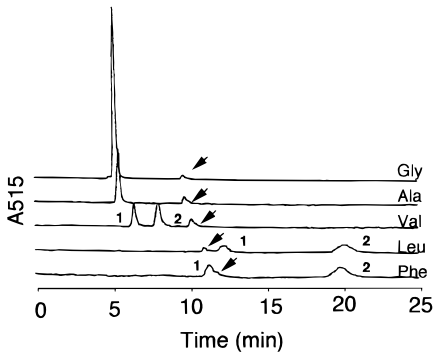

Figure 4. HPLC of the red pigments derived from R1 and amino acids. Column, μ-Bondapak C18; eluent, 50% MeOH; flow rate, 1.0 mL/min. Peaks indicated by arrows were derived from a large amount of unreacted amino acids.

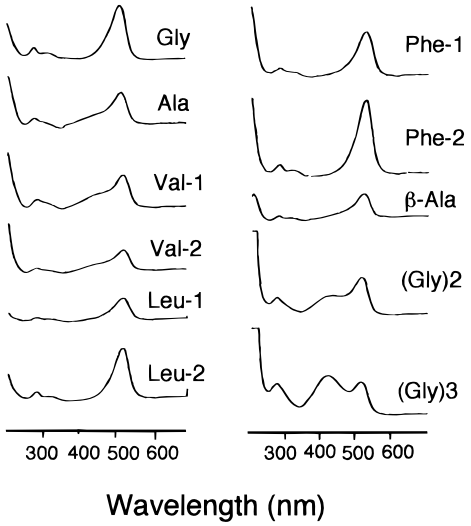

Figure 5. UV−vis spectra of the red pigment separated by HPLC. Red pigments were separated as shown for Figure 4. The spectra were measured directly using an UV−vis detector.

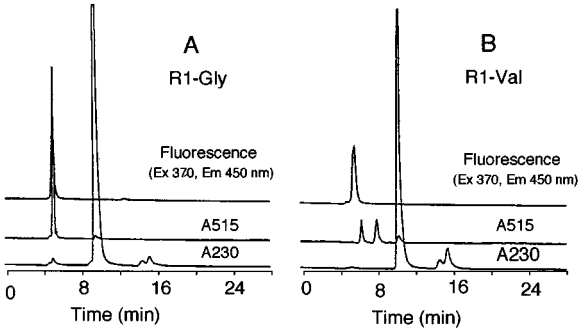

Figure 6. Separation of red pigments and a fluorescent product by HPLC. Conditions of HPLC were the same as for Figure 4.

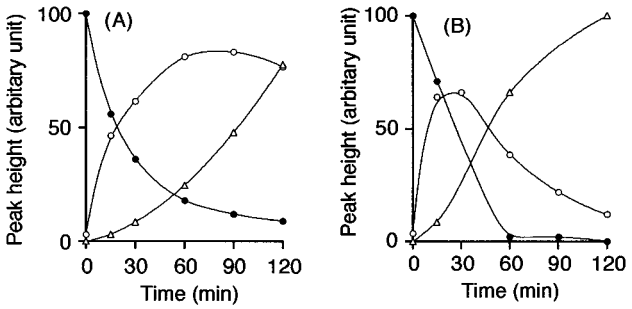

Figure 7. Time course of formation of fluorescent products and red pigment. The conditions of the coloring reaction was the same as for Figure 1. The mixture was incubated at 60 °C (A) and 75 °C (B). The resultant mixture was separated by HPLC, as described for Figure 4: −●−, peak height of unreacted R1; −△−, peak height of fluorescent products; −○−, peak height of red pigment.

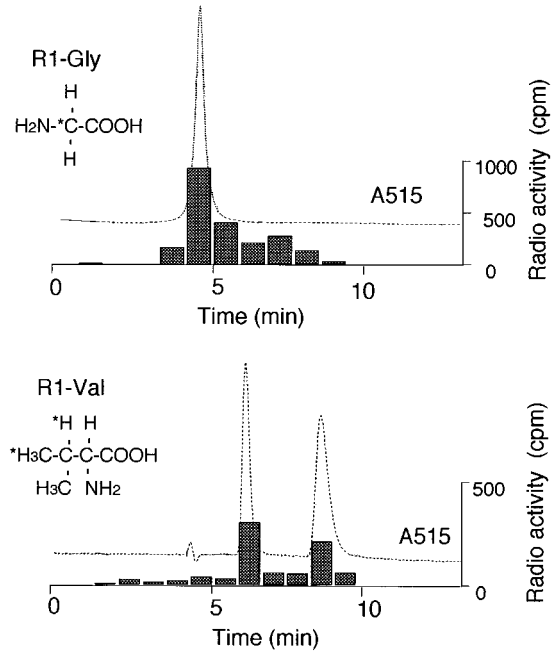

Figure 8. Incorporation of amino acids into the red pigments. The pigments were separated by HPLC, and radioactivity was measured using a liquid scintillation counter.

Incorporation of Amino Acids into the Red Pigments. After removal of unreacted amino acids, the red pigments produced by the reaction with [^{14}C]Gly and [^{3}H]-Val were rechromatographed on a reversed-phase HPLC column. As shown in Figure 8, radioactivity was eluted in synchronization with the red pigments in both assays. Infrared spectra of the purified red pigment derived from R1 and Gly are shown in Figure 9. Strong absorption bands at 1740 cm^{-1} (ketone) and 1690 cm^{-1} (conjugated aldehyde) present in the spectrum of R1 (Nakamura, 1985) disappeared and a strong absorption band at 1630 cm^{-1} that could be assigned to that of $\lambda_{C=N}$ (Montogomery and Day, 1965) appeared alternatively in the spectrum of red pigment. Thus, it can be concluded that both carbonyls participated in the reaction of the red pigment formation and that both carbonyls were responsible for the C=N bond in the pigment.

DISCUSSION

RPSs have the potential for potent red coloring. The molar absorption coefficient of the R1-Gly pigment can

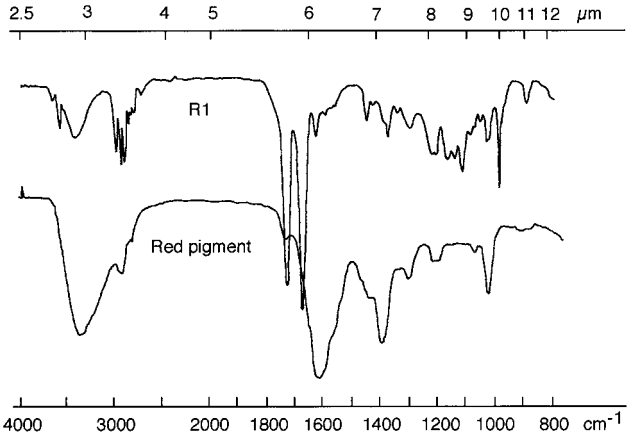

Figure 9. IR spectra of R1 (upper) and the red pigment (lower) derived from R1 and Gly. Spectra of R1 and the pigment were measured in $CHCl_3$ and using a KBr disk, respectively.

be tentatively set at 8500 from the absorption at 515 nm based on two assumptions: all of R1 was reacted to form the red pigment, and one molecule of R1 yielded one molecule of the pigment. The true value is estimated to be much higher than the calculated one since the pigment formed was unstable. Namely, prolonged incubation at a higher temperature or leaving the colored mixture at room temperature for a long time, e.g., overnight, yielded a colorless clear solution, although the red pigments were stable at freezer temperature (−15 °C).

Red coloration that ultimately leads to brownish melanoidins was noted in dried vegetables (Ranganna and Setty, 1968, 1974a,b) or brewed products (Hashiba et al., 1981; Hashiba, 1986). Kurata et al. (1973a,b) studied red coloration with dehydroascorbic acid, an oxidized form of ascorbic acid, and amino acids where the resulting red pigment was identified to be 2,2′-nitrilodi-2(2′)-deoxy-L-ascorbic acid monoammonium salt. This pigment was generated through L-scorbamin, estimated to be an intermediate aminoreductone produced by the Strecker degradation, and was further converted to form the brownish macromolecular compounds (melanoidins). The red pigment had three characteristic absorption bands (λ_{max} 513, 387, and 246 nm) in the UV−vis region. In a series of this coloration reaction, α-amino acids were deaminated and only resultant ammonia was incorporated into the red pigment. As a consequence, the amino acids produced the same compound.

The structure of the red pigment derived from RPS has not been elucidated; however, some coloration characteristics were clarified. As shown in the previous section, almost all of the amino acids tested gave red coloration while the methyl ester of Gly, fatty amines, ammonia, and imines did not. Thus, the presence of one carboxyl group in addition to one amino group in nitrogenous compounds is essential for formation of the red pigments. Both carbonyl groups of RPS are implicated in the red pigment formation, and either or both are responsible for the C=N bond in the pigment, but not for the fluorescence bonding. Deamination of amino acids occurred in the early step of the Maillard reaction, and ascorbic acid−amino acid coloration did not occur in RPS-amino acid red coloration. Large portions of the amino acid's moieties were incorporated and retained in the red pigments. These characteristics are appar-

ently different from red pigments derived from ascorbic acid−amino acid and/or Maillard reactions.

Although the red pigments do not have fluorescence, amino acid-distinctive fluorescent products were produced simultaneously during the red coloration. The fluorescent product derived from R1-Gly showed an excitation maximum at 354 nm and emission maximum at 438 nm, in aqueous solution. This fluorescent characteristic differed from that of the malonaldehyde-Gly condensation product, which has an excitation maximum at 370 nm and an emission maximum at 450 nm (Chio and Tappel, 1969). Thus, cleavage of the five-membered ring of R1 did not occur during the reaction.

ABBREVIATIONS USED

HPLC, high-performance chromatography; MeOH, methanol; R1, a stereoisomer of 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal; RPS, red pigment forming substance.

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LITERATURE CITED

Chio, K. S.; Tappel, A. L. Synthesis and characterization of the fluorescent products derived from malonaldehyde and amino acids. *Biochemistry* **1969**, *8*, 2821−2827.

Cohen, M.P.; Ziyadeh, F. N. Role of Amadori -modified nonenzymatically glycated serum proteins in the pathogenesis of diabetic nephropathy. *J. Am. Soc. Nephrol.* **1996**, *17*, 259−268.

Friedman, M. Food browning and its prevention−An overview. *J. Agric. Food Chem.* **1996**, *44*, 631−653.

Hama, Y.; Maeda, H.; Nakamura, T. Determination of conjugated carbonyls with a prostaglandin-like ring structure in autoxidized lipids. *Nippon Suisan Gakkaishi* **1990**, *56*, 147−152.

Hama, Y.; Itagaki, T.; Nakamura, T. Conjugated carbonyl compounds with a hydroxycyclopentanone ring formed during autoxidation of methyl docosahexaenoate. *J. Jpn. Oil Chem. Soc.* **1992**, *41*, 1111−1114.

Hashiba, H. Oxidative browning of amadori compounds. Color formation by iron with Maillard reaction products. *Dev. Food Sci.* **1986**, *13*, 155−164.

Hashiba, H.; Okuhara, A.; Iguchi, N. Oxygen-dependent browning of soysouce and some brewed products. *Prog. Food Nutr. Sci.* **1981**, *5*, 93−113.

Hayashi, T.; Terao, A.; Ueda, S.; Namiki, M. Red pigment formation by the reaction of oxidized ascorbic acid and protein in a model food system of low moisture content. *Agric. Biol. Chem.* **1985**, *49*, 3139−3144.

Koppanyi, T.; Vinino, A. E.; Veitch Jr., F. P. A reaction of ascorbic acid with α-amino acids. *Science* **1945**, *101*, 541−542

Kurata, T.; Fujimaki, M.; Sakurai, Y. Red pigment produced by the reaction of dehydro-L-ascorbic acid with α-amino acids. *Agric. Biol. Chem.* **1973a**, *37*, 1471−1477.

Kurata, T.; Fujimaki, M.; Sakurai, Y. Red pigment produced by the oxidation of L-scorbamic acid. *J. Agric. Food Chem.* **1973b**, *21*, 676−680.

Montgomery, M. W.; Day, E. A. Aldehyde−amine condensation reaction: A possible fate of carbonyls in food. *J. Food Sci.* **1965**, *30*, 828−832.

Nakamura, T. Red pigment-forming substances from autoxidized linolenate: identification of prostaglandin-like substances. *Lipids* **1985**, *20*, 180−186.

Nakamura, T.; Hama, Y. Prostaglandin-like substances, precursors of red pigment, formed during autoxidation of methyl arachidonate. *J. Agric. Food Chem.* **1988**, *36*, 15−18.

Namiki, M. Chemistry of Maillard reactions; Recent studies on the browning reaction mechanism and the development of antioxidants and mutagens. *Adv. Food Res.* **1988**, *32*, 115−170.

Porta, E. A.; Hartrott, W. S. Lipid pigments in relation to aging and dietary factors (lipofuscins). In *Pigment in Pathology*; Wolman, M., Ed.; Academic Press, Inc.: London, 1988; pp 191−235.

Ranngana, S.; Setty, L. Nonenzymic discoloration of dried cabbage. Ascorbic acid−amino acid interactions. *J. Agric. Food Chem.* **1968**, *16*, 529−533.

Ranngana, S.; Setty, L. Nonenzymic discoloration of dried cabbage. II. Red condensation product of dehydroascorbic acid and glycine ethyl ester. *J. Agric. Food Chem.* **1974a**, *22*, 719−722.

Ranngana, S.; Setty, L. Nonenzymic discoloration of dried cabbage. III. Decomposition products of ascorbic acid and glycine. *J. Agric. Food Chem.* **1974b**, *22*, 1139−1142.

Yin, D. Z. Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. *Free Radical Biol. Med.* **1996**, *21*, 871−876.

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