Relationship between Fluorescence and Antioxidant Activity of Ethanol Extracts of a Maillard Browning Mixture

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ABSTRACT

The relationship between the color and fluorescence of a Maillard browning mixture (0.1M glucose + 0.1M glycine, heated at 100 C) and the antioxidant activity of ethnol extracts of the mixture was investigated. The activity was estimated by comparing the peroxide value development of soybean oil substrates containing the extracts with that of a control. The excitation and emission maxima of the mixture were 365 and 440 nm, and those of the extract 367 and 430 nm. The relative fluorescence (that of 1 μg quinine sulfate/mL 0.01N H_2SO_4 = 100) of the mixture after 16 hr browning was 450, whereas that of the corresponding extract was 175.8. The fluorescence of the mixture increased in parallel to the color (absorbance at 490 nm), which, in turn, was almost proportional to reaction time, except at the earlier stages of browning. The change of the fluorescence with the reaction time at the earlier stages was far greater than that of the absorbance at the same stages. Determination of fluorescence seems to provide a more sensitive method for tracing early browning than that of absorbance. The ethanol extracts from very early stages of the browning exhibited considerable antioxidant activity, and, unlike the absorbance of the mixture or the fluorescence of the extract, the activity of the extract changed very little with the reaction time. It appears that effective antioxidants with almost no color but with considerable fluorescence were already formed at the earlier stages of browning.

INTRODUCTION

Maillard browning reactions have received much attention. They seem more important than any other type of browning reaction, since almost all processed food products contain both reducing sugars and nitrogenous compounds with amino groups. Maillard browning reactions are known to take place almost spontaneously and to proceed faster than any other type of nonenzymatic browning reaction.

Increased stability of heat-processed lard (1) and of cookies with extra sugars (2,3) has been attributed to the formation of antioxidant compound during processing. Anderson et al. (4) reported that stabilities of cereal products were largely dependent upon the antioxidants formed in the cereal during the roasting or puffing processes. Evans et al. (5) and Cooney et al. (6) reported the antioxidant activity of some aminohexose reductones, isolated from the reaction mixtures of hexoses and secondary amines, in soybean oil, cottonseed oil, lard, and shortening.

The antioxidant activity of Maillard browning mixtures has been reported by many workers (7-13). Although the activity has been generally attributed to the amino or nonamino reductones formed in the mixtures (2,4,5,9), the activity of melanoidin pigments -- the final products of the browning mixtures -- has also been reported (7,10).

Krigaya et al. (7) reported that the antioxidant activity of a Maillard browning mixture increased in porportion to the color intensity. However, Hwang and Kim (9) and Lee at al. (11) have shown that the activity of ethanol extracts of Maillard browning mixtures did not increase in proportion to the color intensity.

The fluorescent porperties of Maillard browning mixtures have been studied widely (14-19). Overby and Frost (15) stated that the simultaneous development of color and fluorescence in browning reactions had been known since 1945.

Burton et al. (17) reported that sugars, incubated with an amino function, developed fluorescence much more rapidly and to a greater extent than in its absence. Chio and Tappel (18) isolated fluorescent imino compounds, Schiff's base derivatives, from reaction mixtures of malonaldehyde and amino acids. They reported that the nitrogenous compounds contained the chromophoric group -N=CH-CH=CH-NH- which was probably responsible for the fluorescent properties (18). Adhikari and Tappel (19) reported the chromophoric groups such as $HOOC\text{-}CH_2\text{-}N{=}CH\text{-}CH{=}C\text{-}NH$- or $HOOC\text{-}CH_2\text{-}N{=}CH\text{-}CH{=}C\text{-}OH$ which were believed to be responsible for the fluorescent properties of glucose-glycine browning mixtures.

Although the relationship between the color and antioxidant activity of Maillard browning reactions were occasionally reported (7,9,11,12), there seemed to be little work on the relationship between the fluorescence and antioxidant activity. Therefore, an attempt was made to investigate the relationship between the fluorescence and antioxidant activity of a Maillard browning mixture. It was initially hoped that the investigation would contribute to the elucidation of the nature of antioxidants formed in Maillard browning reactions.

MATERIALS AND METHODS

Substrate Oil and Ethanol Extracts

A commercial edible soybean oil (Dong-Bang Oil Manufacturing Co., Seoul) was used for the antioxidant activity determination of ethanol extracts of a Maillard browning mixture. The peroxide, acid, and iodine values of the oil prior to the determination were as follows: peroxide value: 1.0 ± 0.1 meq/kg oil; acid value: 0.36 ± 0.01; and iodine value: 122.0 ± 1.5.

The peroxide and acid values were determined, respectively, by the AOCS method (20) and the method described by Triebold and Aurand (21). The iodine value was determined by the AOAC-Wijs method (22).

Five hundred mL of a browning mixture (0.1M glucose + 0.1M glycine) was heated in a flask fitted with a reflux condenser at 100 C for 16 hr. Thirty mL of the reaction mixture in three 10-mL portions was taken at successive intervals of 0, ½, 1, 2, 4, 8, and 16 hr. The reaction mixtures were filtered, and kept at 2 C before use.

Each 10-mL portion of the mixture was filtered again, and concentrated at 40 ± 0.1 C. The residue was extracted several times with small portions of absolute ethanol. The combined extract was cooled, and then filtered. Each filtrate was made to 20 mL, and stored at 2 C. before use.

Determination of Absorbance and Fluorescence

The color of the mixture at the successive stages was first visually observed, and then the absorbance (490 nm) of the mixture was determined with a Bausch & Lomb spectrophotometer. Portions of the mixture at later stages were diluted in such a way as diluted mixtures have an absorbance value of about 0.018. The diluted mixtures were also used for the determination of the fluorescence.

As is shown in Figure 1, the maximum excitation and

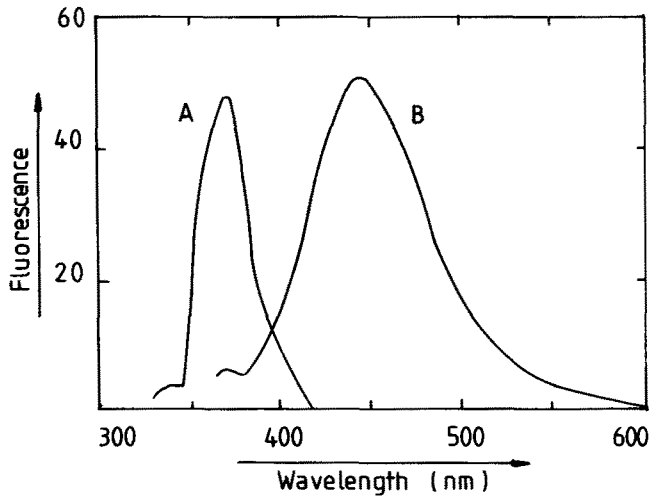

FIG. 1. Excitation (curve A) and emission (curve B) spectra of the 0.1M glucose + 0.1M glycine browning mixture after 4 hr browning. The relative fluorescence of quinine sulfate 1 μg/mL 0.1N H_2SO_4 = 100.

emission wavelengths of the mixture were 365 and 440 nm; those of the extract were 367 and 430 nm. The fluorescence of the mixture at the successive stages was compared with that of 1 μg quinine sulfate/mL 0.01N H_2SO_4, of which fluorescence was arbitrarily taken as 100. The standard quinine sulfate was obtained from Mallinckrodt Chemical Co., St. Louis, MO.

The absorbance and fluorescence of the extract were determined in the same manner as those of the browning mixture.

Determination of Antioxidant Activity

Each 20 mL of the extracts was mixed with 80 g of the soybean oil, and the solvent was removed on a water bath. The oil with each extract was poured equally into three small Petri dishes. Eighty g of the oil without any extract was also placed in three Petri dishes and used as a control. The substrates, i.e., oil portions containing the extracts of the mixture taken at the successive intervals of ½, 1, 2, 4, 8, and 16 hr were termed No. 1, 2, and so on. The substrates and control were stored in an incubator kept at 45.0 ± 1.0 C. for 30 days. The antioxidant activity of the extracts was estimated by comparing the peroxide value development of the substrates with that of the control.

RESULTS AND DISCUSSION

The absorbance and fluorescence of the mixture taken at successive stages of browning are shown in Table I.

The mixture remained almost colorless until the reaction had proceeded for at least 4 hr. The absorbance during this period was almost negligible. The browning of the mixture was slower than expected. The slow browning seemed to be due to the low concentration of the reactants. The absorbance changed nearly in proportion to reaction time after this initial lag period, as is shown in Figure 2. This result seems to be in good agreement with those of Haugaard et al. (23) and Kim (12).

The fluorescence of the mixture also increased almost in proportion to the reaction time after the initial lag period. This result agrees well with those of Tarassuk and Simonson (14) and of Adhikari and Tappel (19).

Although the determination of the absorbance was impossible until the browning had proceeded for at least 4 hr, that of the fluorescence was possible during this period. The determination of fluorescence of a browning mixture

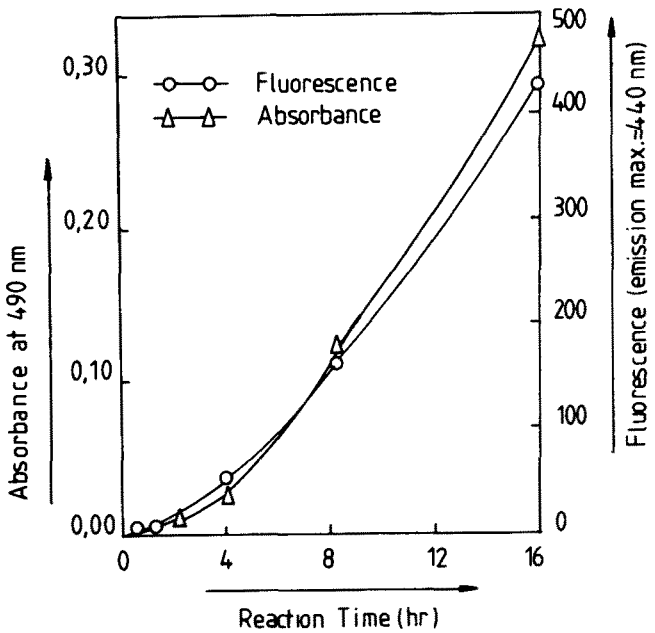

FIG. 2. Variations of the absorbance and fluorescence of the 0.2M glucose + 0.1M glycine browning mixture with reaction time. The relative fluorescence of quinine sulfate 1 μg/mL 0.1N H_2SO_4 = 100.

TABLE I

Variations of Visual Color, Absorbance[a], and Fluorescence[b] of the 0.1M Glucose + 0.1M Glycine Browning Mixture with Reaction Time

Reaction time (hr)	0	½	1	2	4	8	16
Color of browning mixture	color-less	color-less	color-less	color-less	pale yellow	light yellow	orange
Absorbance of browning mixture	0.000	0.000	0.002	0.004	0.018	0.140	0.330
Fluorescence of browning mixture	0.0	4.5	7.1	13.9	51.5	190.0	450.0

[a]Absorbance at 490 nm.

[b]Fluorescence at the emission maximum of 440 nm (excitation maximum = 365 nm) was expressed as relative fluorescence to that of the quinine sulfate standard solution (1 μ/mL 0.1N H_2SO_4 = 100) at room temperature.

seems to be a more sensitive method of tracing early browning than the determination of absorbance of the mixture. Adhikari and Tappel (19) suggested the feasibility of using fluorescence as a quanttative measure of a browning reaction.

Variations of the visual color, absorbance, and fluorescence of the ethanol extract with the reaction time are shown in Table III. The visual color of the extract changed very little with the reaction time. The absorbance increased very slowly with the reaction time.

The absorbance (490 nm) of the browning mixture which had been heated at 100 C for 4, 8, and 16 hr was 0.018, 0.140, and 0.330, whereas that of the corresponding ethanol extracts 0.003, 0.011, and 0.020 (Tables I and II). Lee et al. (11) and Kim (12) reported that brown pigments were not extracted appreciably with absolute ethanol or acetone.

The fluorescence of the extract, on the other hand, increased steadily with the reaction time. The fluorescence of the extracts obtained from the mixture which had been heated for 8 and 16 hr was 84.4 and 175.8, while that of the corresponding mixture was 190.5 and 450.0 (Tables I and II). Unlike the brown pigments, a considerable amount of fluorescent compounds in the browning mixture was extracted with abs.ethanol.

The fact that the nearly colorless ethanol extracts showed considerable fluorescence (cf. Fig. 3) seems to suggest the presence of colorless or nearly colorless fluorescent precursors of brown pigments. Overby and Frost (15)

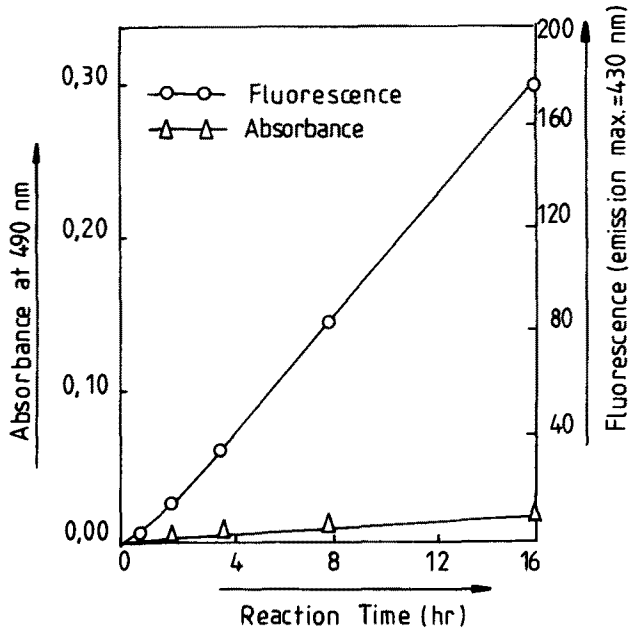

FIG. 3. Variations of the absorbance and fluorescence of the ethanol extracts obtained at successive stages of the 0.1M glucose + 0.1M glycine browning mixture. The relative fluorescence of quinine sulfate 1μg/mL 0.1N H_2SO_4 = 100.

TABLE II

Variations of Visual Color, Absorbance[a], and Fluorescence[b] of the Ethanol Extracts of the 0.1M Glucose + 0.1M Glycine Browning Reaction Mixture with Reaction Time

Sample	Control	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Reaction time (hr)	0	½	1	2	4	8	16
Color of ethanol extracts	color-less	color-less	color-less	color-less	color-less	very pale	pale yellow
Absorbance of ethanol extracts	0.000	0.000	0.000	0.000	0.003	0.011	0.020
Fluorescence of ethanol extracts	0.0	1.2	2.8	16.9	36.3	84.4	175.8

[a]Absorbance at 490 nm.

[b]Fluorescence at the emission maximum of 430 nm (excitation maximum = 367 nm) was expressed as relative fluorescence to that of the quinine sulfate standard solution (1 μg/mL 0.1 N. H_2SO_4 = 100) at room temperature.

TABLE III

Variations of Peroxide Values[a] of the Control and Substrates Containing Equal Amounts of Ethanol Extracts Obtained at Successive Stages of the Browning Mixture with Time

Sample no.	Browning time (hr)	Time[b] (days)			
		0	10	20	30
Control	0	1.3 ± 0.2	12.7 ± 0.1	50.7 ± 0.1	98.2 ± 1.0
No. 1	½	1.4 ± 0.1	4.4 ± 0.1	36.6 ± 0.6	72.5 ± 0.4
No. 2	1	1.3 ± 0.2	3.2 ± 0.2	34.9 ± 0.1	70.8 ± 0.8
No. 3	2	1.5 ± 0.5	3.1 ± 0.4	33.7 ± 0.4	67.1 ± 0.6
No. 4	4	1.4 ± 0.2	3.0 ± 0.3	32.8 ± 0.1	66.8 ± 0.3
No. 5	8	1.4 ± 0.2	2.9 ± 0.4	30.7 ± 0.2	65.8 ± 0.3
No. 6	16	1.4 ± 0.1	2.9 ± 0.1	28.3 ± 0.1	64.3 ± 0.9

[a]Peroxide values were determined by the AOCS method and expressed as meq/kg oil.

[b]All the substrates and control were stored at 45.0 ± 1.0 C.

reported earlier that fluorogens might be precursors of the brown pigments in the browning mixtures of protein hydrolysates and glucose.

Variations of the peroxide values of the control and substrates with the reaction time are shown in Table III. All the extracts exhibited considerable antioxidant activity in the substrates. The extract of the mixture taken after ½ hr showed the least activity, whereas the extract of the mixture taken after 16 hr demonstrated the strongest activity. The difference, however, was not very significant.

Variations of the fluorescence and antioxidant activity with the reaction time are shown in Figure 4. The fluorescence of the extract increased almost in proportion to the reaction time except in the very early stages of browning. The antioxidant activity of the extract did not increase in parallel with the fluorescence. Except for the activity of the extracts of the mixture taken at the very early stages of the browning, the activity remained nearly constant, despite the rapid increase in the fluorescence.

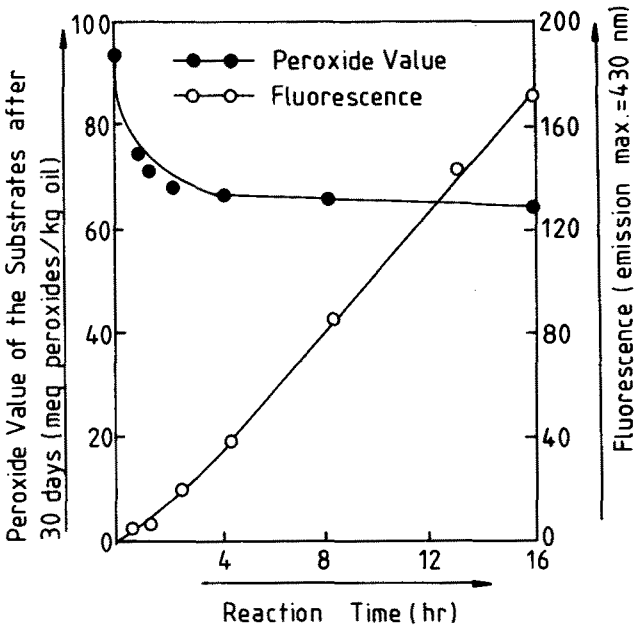

FIG. 4. Relationship between the fluorescence and absorbance of the ethanol extracts obtained at successive stages of the 0.1M glucose + 0.1M glycine browning mixture. The relative fluorescence of quinine sulfate 1 μg/mL 0.1N H_2SO_4 = 100.

The result seems to suggest that active antioxidants with almost no color but with considerable fluorescence were already formed at the very early stages of the browning.

Hodge and Rist (16) suggested that N-glycosides from reducing sugars and amino compounds rearrange spontaneously to form desoxyaminoketoses, which in turn dehydrate spontaneously to nitrogenous reductones. They further stated that the nitrogenous reductones react slowly alone or react rapidly with amino acids to produce fluorescent intermediates and eventually brown pigments. Yamaguchi (8) reported that 3-deoxyxylosone and its browning reaction products did not exhibit any antioxidant activity in lard, but that the reaction products from 3-deoxyxylosone and amines such as N-D-xylosyl-*n*-butylamine showed antioxidant activity. He suggested further that the presence of a nitrogenous group in browning reaction products affect greatly their antioxidant activity. The antioxidant activity of some aminohexose reductones was mentioned earlier in this paper (5,6).

The fluorescence and color of browning reaction products seem related to heterogenous conjugated double-bond systems in the products.

Chio and Tappel (18) reported that the fluorescent properties of N,N′-disubstituted-1-amino-3-iminopropene derivatives R-NH-CH=CH-CH=N-R′ from malonaldehyde-amino acid browning mixtures could be attributed to the chromophoric group -NC=C-CH=N-.

Adhikari and Tappel (19) reported that the chromophoric systems responsible for the fluorescent properties of glucose-glycine mixtures were probably HOOC-CH_2-N=CH-CH=C-NH- or HOOC-CH_2-N=CH-CH=C-OH.

Overby and Frost (15) reported that the colored and fluorescing compounds were not identical in the browning mixtures of glucose and protein hydrolysates. As mentioned earlier, they suggested that fluorogens in the browning mixtures might be precursors of the brown pigments.

The fact that the ethanol extracts with low fluorescence possessed nearly as strong antioxidant activity as the extracts with high fluorescence in the present study seems to suggest the presence of some effective antioxidants which did not have chromophoric systems such as mentioned earlier. Therefore, it seems likely that the structures which contribute to the antioxidant activity are different from the structures which impart the fluorescent character.

The function of phenolic antioxidants has been attributed to the ready formation of stabilized free radicals as a result of the interaction with more reactive free radicals which initiate immediately the chain reactions leading to hydroperoxide formation (24). Structures of phenolic and amine antioxidants such as hydroquinone and diphenylamine are characterized by the presence of active hydrogens at the sites which correspond to benzyl or allyl positions. These active hydrogens are believed to participate readily in free radical H-abstraction reactions.

$$HO\text{-}C_6H_4\text{-}OH\ (\text{-}CH{=}CH\text{-}O\text{-}H) + R\cdot \rightarrow HO\text{-}C_6H_4\text{-}O\cdot(\text{-}CH{=}CH\text{-}O\cdot) + R{:}H$$

$$C_6H_5\text{-}NH\text{-}C_6H_5\ (\text{-}CH{=}CH\text{-}NH\text{-}) + R\cdot \rightarrow C_6H_5\text{-}\dot{N}\text{-}C_6H_5\ (\text{-}CH{=}CH\text{-}\dot{N}\text{-}) + R{:}H$$

Most amino reductones known to possess antioxidant activity (5,6,8) have active hydrogens at heterogenous allyl positions such as -CH-CH=N-CH- or -CH-CH=CH-CH=N-CH- in their molecules. These compounds will probably be able to stabilize more reactive free radicals responsible for the initiation of rapid autoxidation by providing hydrogen radicals through the H-abstraction reactions. It seems likely that, as the formation and decomposition of those compounds which have the active hydrogens at the heterogeneous allyl positions remain in a steady state, the number of the active hydrogens will also remain relatively constant.

Most of the heterogenous double-bond systems formed during the very early stages of browning reactions will spontaneously rearrange into more stable and more highly conjugated fluorescent systems such as the chromophoric systems reported by Chio and Tappel (18) or Adhikari and Tappel (19). These conjugated heterogenous double-bond systems may further polymerize to form highly colored products.

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