Characteristics of the Thiobarbituric Acid Reactivity of Oxidized Fats and Oils

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The thiobarbituric acid (TBA) reactivity of oxidized methyl linoleate, soybean oil, sesame oil, lard, chicken oil and sardine oil was characterized by using four different methods with 0.01% butylated hydroxytoluene (BHT). Optimal pH for the reactivity of most of the oxidized samples was 3-4, and that of some samples was above 5. Introduction of 2 mM *t*-butyl hydroperoxide (*t*-Bu00H) or 0.2 mM ferric ion in the reaction markedly enhanced the reactivity. Introduction of 0.2 mM ethylenediamine tetraacetic acid suppressed the reactivity. The characteristics of the TBA-reactivity of the samples were similar to those of alkadienals or alkenals. The most preferable method for the estimation of the TBA-reactive substances of the oxidized fats and oils was that using solvents at pH 3.5 with introduction of BHT, and *t*-Bu00H or ferric ion.

KEY WORDS: Alkadienals, alkenals, ferric ion, oxidized fats and oils, TBA-reactivity, *t*-butyl hydroperoxide.

Oxidized fats and oils produce the red 1:2 malonaldehyde-thiobarbituric acid (TBA) pigment on heating with TBA in an acidic medium, and malonaldehyde and its precursors have been long considered to be responsible for the pigment formation (1-4). Ohkawa *et al.* (5) and Asakawa and Matsushita (6) demonstrated that the TBA-reactivity of pure fatty ester hydroperoxides is the highest, at pH 3-4. In addition, it was found that ferric and ferrous ions enhance the reactivity (6,7). Since the TBA-reactivity of malonaldehyde is not enhanced at that pH value and by the metal ions, this enhancement was tentatively ascribed to the increased decompostion of the hydroperoxides into malonaldehyde (6).

Recent investigations have demonstrated that alkadienals and alkenals generated in lipid oxidation are prone to produce the same pigment in substantial yields (8-13), and the characteristics of the TBA-reactivity of these aldehydes are different from those of malonaldehyde. The reactivity of alkadienals and alkenals is the highest at pH 3-4 and above 5, respectively (12), and is enhanced by *tert*-butyl hydroperoxide (*t*-Bu00H) (9-12). Furthermore, the TBA-reactivity of 2-hexenal is enhanced by ferric ion (13).

In a previous paper (14), we have suggested that the major TBA-reactive substances in oxidized fats and oils may be due to aldehydes other than malonaldehyde, since the TBA-reactivity of the samples was much higher than that due to the contents of malonaldehyde and its precursors. In the present communication, we characterized the TBA-reactivity of oxidized fats and oils by using several different assay variations, pH values, *t*-Bu00H, ferric ion and ethylenediamine tetraacetic acid (EDTA) to identify the major TBA-reactive substances, and offered mechanistic consideration for the TBA-reactivity of oxidized fats and oils.

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EXPERIMENTAL PROCEDURES

Materials. TBA and 2,4-nonadienal were obtained from Wako Pure Chemical Industries (Osaka, Japan). 2-Hexenal, 2,4-hexadienal, tetramethoxypropane and methyl linoleate were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). *t*-Butyl hydroperoxide (*t*-Bu00H, 70% in water) was purchased from Sigma Chemical Co. (St. Louis, MO). Butylated hydroxytoluene (BHT) was from Nikki Universal Co. (Tokyo, Japan). Malonaldehyde sodium salt was prepared from tetramethoxypropane (15). Soybean oil was a product of Showa-Sangyo Co. (Tokyo, Japan), and sesame oil was Japan Pharmacopoeia grade. Subcutaneous lipids of hog (lard) and chicken, as well as whole lipids of sardine meat, were extracted and prepared as described (16).

Oxidized fats and oils. Methyl linoleate, soybean oil, sesame oil, lard and chicken oil were oxidized at 98°C by the active oxygen method (AOM) (17). Sardine oil was oxidized by ultraviolet light (UV)-irradiation (16). Hydroperoxide contents of the oxidized samples were determined by peroxide value (meq/Kg) (18).

TBA test. The TBA test was carried out directly on the samples by four different variations: method A, in 2% acetic acid (14); method B, by Buege and Aust (19); method C, by Uchiyama and Mihara (20); and method D, by Ohkawa *et al.* (5). The major differences in these methods are the solvents employed and, thus, the pH values of the reaction mixtures. All the reactions were performed by the two-step mode which involves an initial treatment at 5°C and subsequent heating at 100°C (9-11,14). *t*-BuOOH (2 mM) (9-12,14), 0.2 mM ferric chloride (6,13) and 0.2 mM ethylenediamine tetraacetic acid (EDTA) (7,13) were introduced in the reaction. In order to prevent undesirable lipid oxidation during the assay, 0.01% BHT (1,6,14,21) was introduced in the reaction. Absorption spectra of the reaction mixtures or the extracts were measured on a Shimadzu UV-240 visible (Kyoto, Japan) or a Hitachi U-2000 spectrophotometer (Tokyo, Japan). The amount of red pigment was determined by absorbance at 532 nm and the extinction coefficient of the 2:1 TBA-malonaldehyde pigment at 532 nm:156000 (4).

Method A. In a 13-mL screw-cap tube, 5.0 mL of 0.40% TBA in water, 0.1 mL of a mixture of BHT (final concentration 0.01%) and sample in glacial acetic acid, and 10 μL of *t*-BuOOH (final concentration 2.0 mM) in glacial acetic acid were placed (in that order). The pH value of the reaction mixture was about 2.1. The mixture was kept at 5°C for 60 min and then heated at 100°C for 20 min. The mixture was extracted with 2.0 mL of chloroform to remove the oil.

Method B. In a test tube, 1.0 mL of water, 2.0 mL of 0.375% TBA in 15% trichloroacetic acid-0.25 N hydrochlo-

ric acid, 50 μL of a mixture of BHT (0.01%) and sample in glacial acetic acid, and 10 μL of *t*-BuOOH (2.0 mM) in glacial acetic acid were placed, in that order. The pH value of the reaction mixture was about 0.7. The mixture was kept at 5°C for 60 min and then heated at 100°C for 15 min. The mixture was extracted with 2.0 mL of chloroform to remove the oil.

Method C. The following were placed in a test tube in this order: 0.5 mL of water, 3.0 mL of 1% phosphoric acid, 1.0 mL of 0.6% TBA in water, 50 μL of a mixture of BHT (0.01%) and sample in glacial acetic acid, and 10 μL of *t*-BuOOH (2.0 mM) in glacial acetic acid. The pH value of the reaction mixture was about 1.8. The mixture was kept at 5°C for 60 min and then heated at 100°C for 45 min. Red pigment was extracted with 4.0 mL of *n*-butanol.

Method D. These solutions were placed in a test tube in the following order: 0.8 mL of water [or water containing ferric chloride (0.2 mM) or EDTA (0.2 mM)], 0.2 mL of 8.1% sodium dodecylsulfate, 1.5 mL of 20% acetic acid solution adjusted at pH 2-5.5 with 10 N NaOH, 1.5 mL of 0.8% TBA in water, 50 μL of a mixture of BHT (0.01%) and sample in glacial acetic acid, and 10 μL of *t*-BuOOH (2.0 mM) in glacial acetic acid. The mixture was kept at 5°C for 60 min and then heated at 100°C for 60 min. Red pigment was extracted with 1.0 mL of water and 5.0 mL of *n*-butanol-pyridine (15:1).

TABLE 1

Amount of Red Pigment from Oxidized Methyl Linoleate and Soybean Oil in Four Different Variations of the TBA Assay with 0.01% BHT[a]

Oxidized methyl linoleate (peroxide value 1600 meq/Kg)		
	Red pigment (nmol/mg sample)	
Method	-*t*-BuOOH	+ 2 mM *t*-BuOOH
A	10.07	13.46
B	2.94	4.80
C	2.70	3.78
D (pH 3.5)	24.20	39.25
Oxidized soybean oil (peroxide value 710 meq/Kg)		
	Red pigment (nmol/mg sample)	
Method	-*t*-BuOOH	+ 2 mM *t*-BuOOH
A	4.79	5.48
B	2.68	3.12
C	3.36	3.43
D (pH 3.5)	11.04	16.03

[a]All the tests were performed using 1.5 mg of the samples.

RESULTS

The TBA test of oxidized methyl linoleate and soybean oil with and without *t*-BuOOH was performed in the four different variations in the presence of 0.01% BHT—method A in 2% acetic acid (14); method B by Buege and Aust (19); method C by Uchiyama and Mihara (20); and method D at pH 3.5 by Ohkawa *et al.* (5) (Table 1). Major differences of these variaitons were those of solvents employed, and thus, the pH values of the reaction mixtures. Both the oxidized oils without *t*-BuOOH produced different amounts of the pigment in the variations—the highest yield was obtained in method D at pH 3.5 (Table 1, left column). Enhancement by *t*-BuOOH was observed in all the variations (Table 1, right column). The differences of the TBA-reactivity of these oils in the variations were similar to those of 2,4-hexadienal, although not identical to those of tetramethoxypropane (12).

In order to assess autoxidation of lipid samples during the TBA test, the time-course of the TBA-reactivity of unoxidized methyl linoleate and soybean oil in method D at pH 3.5 in the absence and presence of BHT was followed (Fig. 1A and B). In the absence of BHT, the amount of the pigment produced in the reaction mixtures with and without *t*-BuOOH progressively increased and did not reach plateaus, indicating that substantial autoxidation took place. In the presence of 0.01% BHT, these increases were effectively prevented, even with *t*-BuOOH. BHT was also effective in the reaction of these oils with 0.2 mM ferric ion (data not shown). Hence, incorporation of 0.01% BHT was required to prevent undesirable autoxidation of the samples during the test.

pH Dependence of the TBA-reactivity of standard aldehydes with and without *t*-BuOOH, ferric ion and EDTA in method D was investigated (Fig. 2). The TBA reactivity of tetramethoxypropane and malonaldehyde sodium salt was almost constant between pH 2-4 and was lower above pH 5 (Fig. 2A, solid line). *t*-BuOOH slightly decreased the reactivity throughout the pH ranges (Fig. 2A, dotted line), which may be due to the decomposition of malonaldehyde by the hydroperoxide, as has been demonstrated previously, (22,23). In addition, ferric ion (Fig. 2A, chained line) and EDTA (Fig. 2A, line with small dots) gave little effect on the reactivity. The yield of the pigment from tetramethoxypropane was almost quantitative under the reaction conditions below pH 4.

The greater pH dependence of the TBA-reactivity of alkenals and alkadienals is shown in Fig. 2B-D. The reactivity of 2-hexenal was maximal above pH 5 (Fig. 2B, solid line), and that of 2,4-hexadienal and 2,4-nonadienal was maximal at pH 3-4 (Fig. 2C and D, solid line). *t*-BuOOH promoted the reactivity of these aldehydes below

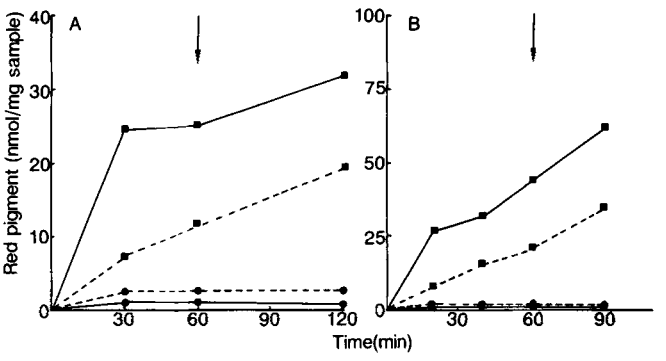

FIG. 1. Autoxidation of unoxidized methyl linoleate (A), and soybean oil (B), during the TBA test period in method D at pH 3.5. The amount of red pigment formed from 1.0 mg of the sample in the absence (■), and presence (●), of 0.01% BHT was plotted. Arrows indicate the standard reaction period of method D. With none (——), and with 2 mM *t-BuOOH* (----).

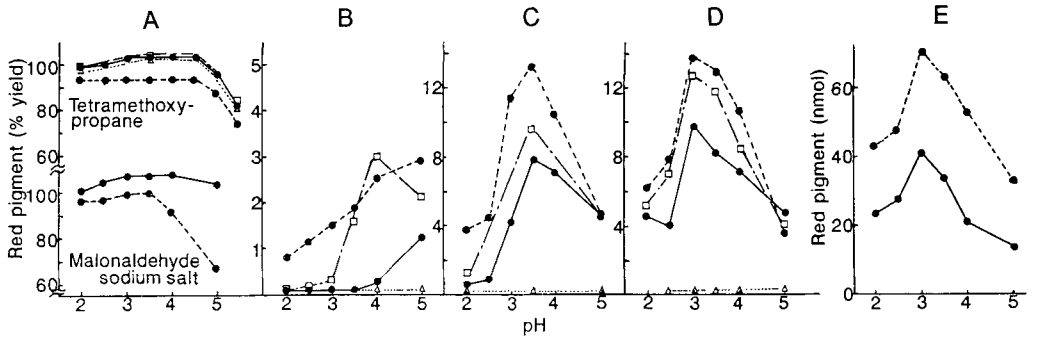

FIG. 2. Effect of pH, *t*-BuOOH, ferric ion and EDTA on the TBA-reactivity of standard aldehydes in method D with 0.01% BHT. A: Tetramethoxypropane or malonaldehyde sodium salt (20 nmol). B: 2-hexenal (0.84 μmol). C: 2,4-hexadienal (0.44 μmol). D: 2,4-nonadienal (0.33 μmol). E: A mixture of 2-hexenal (0.84 μmol) and 2,4-nonadienal (0.33 μmol). With none (—●—), 2 mM *t*-BuOOH (---○---), 0.2 mM ferric chloride (—□---), and 0.2 mM EDTA (---△---).

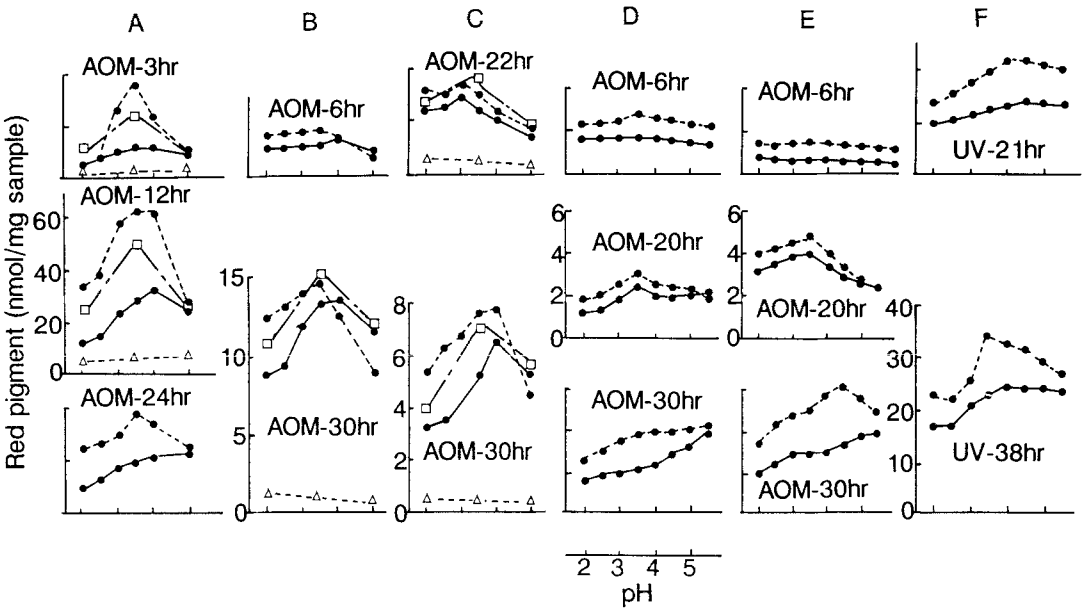

FIG. 3. Effect of pH, *t*-BuOOH, ferric ion and EDTA on the TBA-reactivity of oxidized fats and oils in method D with 0.01% BHT. A: oxidized methyl linoleate; AOM-3 hr (peroxide value 890 meq/Kg), AOM-12 hr (1620) and AOM-24 hr (1010). B: oxidized soybean oil; AOM-6 hr (220) and AOM-30 hr (710). C: oxidized sesame oil; AOM-22 hr (490) and AOM-30 hr (800). D: oxidized lard; AOM-6 hr (90), AOM-20 hr (260) and AOM-30 hr (710). E: oxidized chicken oil; AOM-6 hr (60), AOM-20hr (300) and AOM-30 hr (940). F: oxidized sardine oil; UV-21 hr (530) and UV-38 hr (740). Each sample of 0.2–2.0 mg was subjected to the test. With none (—●—), 2 mM *t*-BuOOH (---●---), 0.2 mM ferric chloride (—□---), and 0.2 mM EDTA (---△---).

pH 4 (Fig. 2B-D, dotted line). In addition, ferric chloride enhanced the reactivity of these aldehydes below pH 4 (Fig. 2B-D, chained line). EDTA completely suppressed the reactivity of these aldehydes throughout the pH ranges (Fig. 2B-D, line with small dots). The TBA-reactivity of these aldehydes was not significant in the presence of both EDTA and *t*-BuOOH (data not shown). The yield of the pigment from 2-hexenal was about 3%, and those from 2,4-hexadienal and 2,4-nonadienal were about 13% under the optimal conditions. When 2-hexenal and 2,4-nonadienal were mixed in a 2.5:1 ratio, the TBA-reactivity with and without *t*-BuOOH was maximal at pH 3 (Fig. 2E), which was similar to that of 2,4-nonadienal alone (Fig. 2D).

pH Dependence of the TBA-reactivity with method D of oxidized methyl linoleate, soybean oil, sesame oil, lard, chicken oil and sardine oil with different stages of autoxidation was investigated (Fig. 3). The pH dependence was different with the samples and the stages of their autoxidation (Fig. 3, solid line). The optimal pH for most samples was 3–4, and for some samples was above 5. *t*-BuOOH enhanced the reactivity of most samples (Fig. 3, dotted line). Ferric ion enhanced the reactivity of oxidized methyl linleate, soybean oil and sesame oil (Fig. 3, chained line), and EDTA suppressed the reactivity of these oils almost completely (Fig. 3, line with small dots). The TBA-reactivity of these oils was not significant in the presence of both EDTA and *t*-BuOOH (data not shown). The characteristics of the TBA-reactivity of these oxidized fats and oils were similar to those of alkadienals

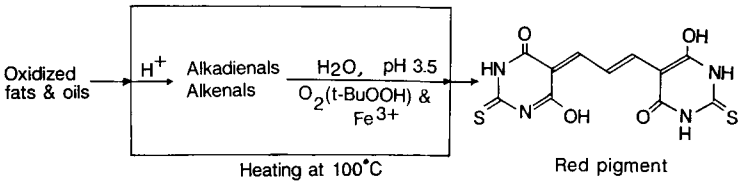

FIG. 4. The mechanism of the TBA reaction of oxidized fats and oils in method D with BHT.

and/or alkenals, and essentially different from those of tetramethoxypropane.

DISCUSSION

In a previous paper (14), it has been demonstrated that oxidized fats and oils release only a small amount of malonaldehyde and substantial amounts of other aldehydes, and that their TBA-reactivity is much higher than that due to their malonaldehyde contents. It has been suggested that aldehydes other than malonaldehyde contribute to the TBA-reactivity. It was found in the present investigation that the TBA-reactivity of various oxidized fats and oils was greatly dependent on the pH values of the reaction mixtures. The optimal pH was 3–4 or above 5. Introduction of *t*-BuOOH or ferric ion in the reaction markedly enhanced the reactivity of all the samples. Introduction of EDTA completely suppressed the reactivity even in the presence of *t*-BuOOH. The characteristics of the TBA-reactivity of the oxidized fats and oils were similar to those of alkadienals and/or alkenals.

The pH dependence of the TBA-reactivity of pure fatty ester hydroperoxides was reported earlier (5,6), and the reactivity has been ascribed to that of the hydroperoxides themselves (5). The effect of ferric ion and EDTA on the reactivity of the hydroperoxides was also known (6,7). At that time, it was assumed that ferric ion promoted the decomposition of the hydroperoxides into malonaldehyde, and EDTA trapped the metal ion contaminated in the reaction mixtures to prevent the decomposition (6,7). However, judging from the present results, it looks more likely that the pH dependence of the reactivity of the oxidized fats and oils was due to the released alkadienals and/or alkenals, and enhancement of the reactivity by *t*-BuOOH and ferric ion was due to that of these aldehydes. The major TBA-reactive substances from the oxidized fats and oils may be reasonably ascribed to alkadienals, alkenals and those released from the samples under the test conditions.

The large difference in the TBA-reactivity of oxidized fats and oils in the four variations of the assay (Table 1) may be due to the reactivity of alkadienals and alkenals. Method D at pH 3.5 proposed by Ohkawa *et al.* (5) may be the most preferable method for the assay of the TBA-reactive substances of the oxidized fats and oils. In this assay method, introduction of BHT was requisite to prevent undesirable autoxidation during the assay. For effective pigment development, incorporation of *t*-BuOOH and ferric ion was preferable. If the assay were performed by use of the solvents and the reagents free of contaminated ferric ion, the TBA-reactivity of these fats and oils may be much lower. If the assay were performed in the absence of dissolved oxygen or any oxidants, the reactivity may be suppressed.

The mechanisms for the TBA-reactivity of the oxidized fats and oils in method D at pH 3.5 in the presence of BHT may be postulated in Figure 4. Under the TBA test conditions, the oxidized fats and oils may release alkadienals and alkenals by heat in the mildly acidic water, and the released alkadienals and alkenals may be reacted with TBA to form the red 1:2 malonaldehyde-TBA pigment by heat in the mildly acidic water with dissolved oxygen, more efficiently, *t*-BuOOH, and with ferric ion.

We have reported recently that the TBA-reactive substances from liver homogenate may be alkadienals (12). The present results indicated that the TBA-reactive substances from the oxidized fats and oils may be alkadienals and/or alkenals. In contrast, we could not identify the TBA-reactive substances from blood plasma, which may be compounds other than malonaldehyde, alkadienals and alkenals (24). Hence, the TBA-reactive substances may be different with the samples from different origin. Before estimation of the TBA-reactivity of samples, it is important to characterize the TBA-reactivity and realize what substances are measured in the TBA test.

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