Effects of Alkylamines and PC on the Oxidative Stability of Soybean Oil TAG in Milk Casein Emulsions

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ABSTRACT: The effects of alkylamines and PC on the copper-catalyzed oxidation of soybean oil TAG were studied in milk casein emulsions. Stearylamine showed an antioxidant effect in casein emulsions in the presence of PC, whereas dicetylphosphate acted as a prooxidant. The antioxidant or prooxidant effect could be explained by the electrostatic repulsion or attraction between positively charged stearylamine or negatively charged dicetylphosophate and positively charged copper ion at the interface, respectively. On the other hand, these effects were not observed in the absence of PC, suggesting the importance of PC for charged components to show their activities at the interface. Other types of alkylamines—spermine, spermidine, and putrescine—also inhibited the oxidation of soybean oil TAG emulsified with casein in the presence of PC. The antioxidant effects of these natural polyamines were higher than that of stearylamine. PC molecular species also affected soybean oil TAG oxidation in emulsion. The oxidative stability of soybean oil TAG increased in the emulsion with PC containing oleic acid.

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KEY WORDS: Alkylamine, emulsion, milk casein, oxidative stability, phosphatidylcholine.

Metal catalysts, such as iron and copper, are recognized as factors in accelerating lipid oxidation in food emulsion systems, particularly in milk and dairy products (1,2). In milk, copper is the most important catalyst for the development of oxidative flavors, although it occurs naturally in milk at a lower concentration (20–40 μg/L) than iron (100–250 μg/L) (3). The generally accepted mechanism of initiation of lipid oxidation in these food systems involves the catalytic action of metal at the dispersed fat globule membrane–water interface. Therefore, metal chelators such as EDTA reduce the development of oxidized flavors in such systems, but the use of these additives is not allowed in the United States and other countries.

On the other hand, an electrical charge on the membrane significantly influences the effectiveness of positively charged metal ions (4). Mei *et al.* (5,6) reported that iron-catalyzed oxidation of corn oil in emulsion was accelerated by a negatively charged emulsifier but inhibited by a positively charged emulsifier. These results suggest that metal-catalyzed lipid oxidation in emulsion systems also may be inhibited by the presence of positively charged components at the oil–water interface.

This paper describes the antioxidant effect of alkylamines as positively charged components on the aqueous oxidation of soybean oil TAG in the presence of copper catalyst in a milk casein emulsion. In milk, lipids are dispersed as globules covered with membranes composed of milk proteins and phospholipids. Therefore, we also examined the effects of PC, including milk PC, on the oxidative stability of soybean oil TAG in the emulsion.

MATERIALS AND METHODS

Sample lipids. Soybean oil was obtained from Kanto Chemical Co. (Tokyo, Japan). The oil (25 g) was passed through a column packed with a 1:1 *n*-hexane slurry mixture (w/w) of activated carbon and Celite 545 to remove tocopherols (7). The recovered oil (24 g) was refined on a silicic acid column by eluting with a diethyl ether/*n*-hexane solution just before use. The fraction eluted with diethyl ether/*n*-hexane (10:90 and 20:80, vol/vol) (20 g) was used as the oil sample for oxidation. The purified oil sample contained no tocopherol as determined by HPLC (8) and gave only a single spot corresponding to TAG on the thin layer-chromatogram with normal-phase silica plates (Merck, Darmstadt, Germany) developed with diethyl ether/*n*-hexane/acetic acid (40:60:1, by vol). The PV of each sample was less than 1.0 as determined by the AOCS Official Method (9). The FA composition of the soybean oil TAG was determined by GC after conversion of fatty acyl groups in TAG to their methyl esters by heating in a sealed tube at 90–100°C for 1 h with 7% BF_3-MeOH in methanol under nitrogen. GC analysis was performed on a Shimadzu GC-14B (Shimadzu Seisakusho, Kyoto, Japan) equipped with an FID and a capillary column [Omegawax 320 (30 m × 0.32 mm i.d.); Supelco, Bellefonte, PA]. The FA composition of soybean oil TAG is shown in Table 1.

Materials. Milk casein was obtained from Sigma Chemical Co. (St. Louis, MO). As alkylamines, we used stearylamine ($CH_3(CH_2)_{16}CH_2NH_2$), spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$), spermidine ($NH_2(CH_2)_3NH(CH_2)_4NH_2$), and putrescine ($NH_2(CH_2)_4NH_2$). Stearylamine was obtained from Wako Pure Chemical Ind. (Osaka, Japan); the other three alkylamines were obtained from Sigma Chemical. The negatively charged component, dicetylphosphate, also was obtained from Sigma Chemical. Copper sulfate ($CuSO_4$)

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TABLE 1
FA Composition of Soybean Oil TAG and Milk PC

FA (wt%)	Soybean oil TAG	Milk PC
14:0	0.1	7.7
16:0	10.2	31.5
18:0	3.1	5.8
16:1n-7	0.1	2.6
18:1n-9	18.7	30.3
18:2n-6	57.0	8.2
18:3n-3	8.5	1.0

as an oxidation inducer was obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

1,2-Dipalmitoyl-*sn*-glycero-3-phosphate (1,2-diPA-PC) was obtained from Wako Pure Chemical. 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphate (1-PA-2-LA-PC), 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphate (1-PA-2-AA-PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (1-PA-2-OA-PC), and 1,2-dioleoyl-*sn*-glycero-3-phosphate (1,2-diOA-PC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 1-Palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphate (1-PA-2-DHA-PC) was obtained from Sigma Chemical. Milk PC was kindly donated by Snow Brand Milk Products (Saitama, Japan). Each PC gave only a single spot by TLC (10) and contained no tocopherols or peroxides, as seen by HPLC (8,11). The FA composition of milk PC was analyzed by capillary GC after converting the fatty acyl groups in the PC to their methyl esters with 7% BF_3-MeOH. The main FA identified in milk PC are listed in Table 1.

Aqueous oxidation. Soybean oil TAG (250 mg) was mixed homogeneously in chloroform. Each PC or negatively (dicetylphosphate) or positively (stearylamine, spermine, spermidine, and putrescine) charged component was added by dissolving it in the chloroform solution. After removing the chloroform by gently sweeping with nitrogen, 0.05 M phosphate buffer (pH 7.4 at 37°C; 45 mL) containing milk casein was added to the mixture. The emulsion was obtained by homogenizing the mixture for 8 min with Physcotron NS-50 (Nition Seisakusho Co., Chiba, Japan), and the oxidation was initiated by adding a $CuSO_4$ aqueous solution (5 mL) to the substrate solution (45 mL). Final concentration of each component in the reaction mixture was as follows: TAG: 0.5% (wt/vol); protein: 0.2% (wt/vol); $CuSO_4$: 0.1 or 1 mM; charged component: 0.1 mM; PC: 0.5 mM.

Analysis of aqueous oxidation. Oxidative stability was evaluated by analyzing oxygen consumption. For continuously monitoring oxygen uptake by the oxidation of lipids in the solution, a model 5300 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) was used. As soon as the $CuSO_4$ solution had been added to the substrate solution, the reaction vessel was charged with 3 mL of the reaction solution and the concentration of dissolved oxygen in the solution was measured. The concentration of $CuSO_4$ in the reaction solution was 1 mM. The oxidation was carried out at least three times for each emulsion sample. For each determination there was a slight difference in the oxidation rate, but the order of the oxidative stability of different emulsion samples used in the present study was unchanged and there was no siginificant difference in the oxidation rate between these samples.

Oxidation was also followed by a decrease in the substrate during oxidation with GC. The reaction solution (10 mL), containing 0.1 mM of $CuSO_4$, was incubated in a flat-bottomed glass tube (30 mL, 2.6 cm i.d.) in the dark at 37°C. After a timed period of incubation, the whole reaction mixture was extracted with chloroform/methanol (2:1, vol/vol). The extract was washed with water, dried over anhydrous sodium sulfate, concentrated *in vacuo*, and methylated with 7% BF_3-MeOH in methanol. The FAME were purified by silicic acid column chromatography and then subjected to GC. The decrease in each PUFA by oxidation was evaluated from the change in its peak ratio to palmitic acid. The procedures for methylation and GC analysis were the same as those described above. Three separate experiments were done for each sample, and the data were expressed as mean + SD.

RESULTS AND DISCUSSION

When soybean oil TAG were oxidized in an emulsion dispersed with milk casein in the presence of Cu^{2+} as an oxidation inducer (Fig. 1), both kinds of charged components, negatively dicetylphosphate and positively stearylamine, had little effect on the oxidation rate. Since both components have long-chain alkyl groups in their molecules, they are fat soluble, and most of these molecules would be present in the oil droplet interior. Therefore, it might be difficult for these components to change the electrical charge of the interface and thus they would have little effect on lipid oxidation under these experimental conditions.

On the other hand, both charged components showed significant effects on the oxidative stability of soybean oil TAG in a milk casein emulsion in the presence of 0.5 mM of 1-PA-2-LA-PC + 1,2-diPA-PC (1:1 molar ratio). As shown in Figure 2, dicetylphosphate promoted the oxidation of soybean oil TAG in the emulsion because of the electrostatic attraction between negatively charged dicetylphosphate and positively charged copper ion at the interface. Oxidation might be accelerated by the increase in copper–lipid interactions at the interface. In contrast, stearylamine acted as an antioxidant in the aqueous oxidation of soybean oil TAG (Fig. 2). This may also be explained by the electrostatic repulsion between positively charged stearylamine and copper ion. Comparison of the results in Figures 1 and 2 suggests that the presence of PC is important if charged components are to exhibit their effects at the interface. PC will make a fat globule membrane with casein at the interface. Charged components may interact with the ionic PC molecule, and more charged components could exist at the interface in the presence of PC.

Other types of alkylamines such as spermine, spermidine, and putrescine showed an inhibitory effect on the oxidation of soybean oil TAG in the presence of three kinds of PC. Table 2 shows the oxidation rate of soybean oil TAG in a casein emulsion, indicated as the time in hours to lose 20% of dissolved oxygen by oxidation. The rate decreased in the

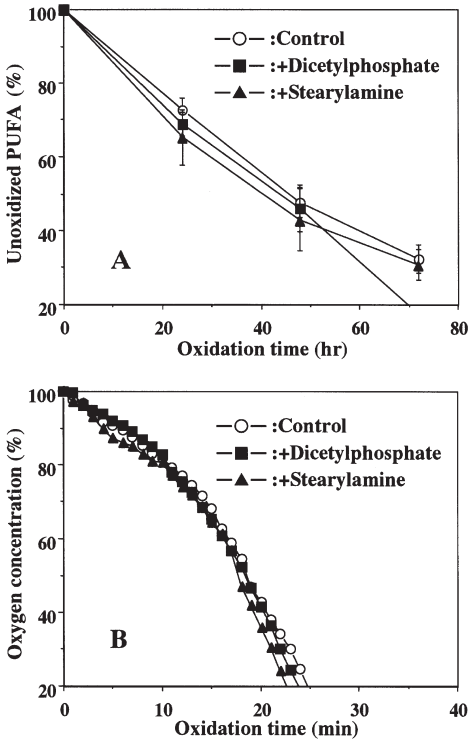

FIG. 1. Effects of negatively charged dicetylphosphate and positively charged stearylamine on the oxidative stability of soybean oil TAG in casein emulsions without PC. Oxidation was followed by a decrease in unoxidized PUFA (A) and in dissolved oxygen concentration (B).

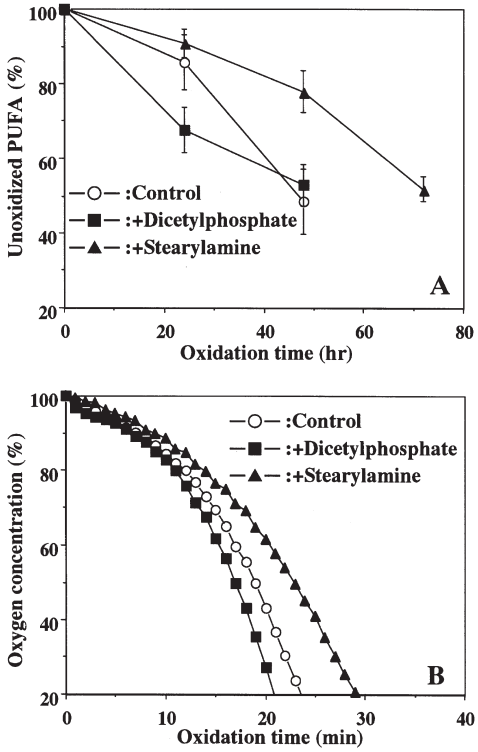

FIG. 2. Effects of charged components on the oxidative stability of soybean oil TAG in casein emulsions in the presence of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphate (1-PA-2-LA-PC) + 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (1,2-diPA-PC) (1:1 molar ratio). Oxidation was followed by a decrease in unoxidized PUFA (A) and in dissolved oxygen concentration (B).

presence of added alkylamines in every case. The effects of these natural amines were higher than that of stearylamine in all emulsions used in Table 2. The difference was especially significant in the emulsions with 1-PA-2-AA-PC + 1,2-diPA-PC and 1-PA-2-DHA-PC + 1,2-diPA-PC. These natural polyamines possess more amino groups and show stronger basicity than stearylamine. Saito *et al.* (12) reported on the antioxidant activity of basic alkylamines in bulk phase and found a good correlation between their activities and basicities. They also demonstrated that the effect of alkylamines would be derived from their decomposition or reduction activity on lipid hydroperoxides.

Table 2 also shows the effect of alkylamines and dicetylphosphate on the oxidation of soybean oil TAG in a casein emulsion in the presence of milk PC. The antioxidant or prooxidant activity of these charged components was also observed. Furthermore, when the oxidation rate of the control system (without charged components) was compared with those of controls in casein emulsions with other polyunsaturated PC (Table 2), the former was oxidatively more stable than the latter. In the present emulsion systems with protein and PC, oxidation occurs initially in the PC fraction associated with the fat globule membrane, followed by the main TG fraction (3). As shown in Table 1, the main FA of milk PC were palmitic acid (PA) and oleic acid (OA), and these FA are relatively stable to oxidation. Therefore, the abundance of these saturated and monounsaturated FA in milk PC would be strongly correlated with the higher oxidative stability of soybean oil TAG in the emulsion with milk PC.

Figure 3 shows the effects of three kinds of synthesized PC containing PA and OA on the oxidative stability of soybean oil TAG in casein emulsions. These PC are presumed to be the main PC molecular species in milk PC. The stability of soybean oil TG in the emulsion with PC containing OA (1,2-diOA-PC or 1-PA-2-OA-PC) was much higher than that in emulsions with other kinds of synthetic PC, i.e., 1-PA-2-LA-PC + 1,2-diPA-PC, 1-PA-2-AA-PC + 1,2-diPA-PC, and 1-PA-2-DHA-PC + 1,2-diPA-PC (Table 2). This result showed the contribution of these PC molecules containing OA to the higher stability of soybean oil TAG in the emulsion with milk PC.

On the other hand, the oxidative stability of soybean oil TAG in an emulsion with 1,2-diPA-PC was lower than that in emulsions with the other two kinds of PC containing PA and/or OA (Fig. 3), and was almost the same as that in emulsions with 1-PA-2-LA-PC + 1,2-diPA-PC and 1-PA-2-DHA-PC + 1,2-diPA-PC (Table 2), although 1,2-diPA-PC and 1-PA-2-DHA-PC were oxidatively the most and least stable PC used in the present study, respectively. These results suggest the involvement of other factors than the degree of unsaturation in the oxidative stability of lipids in the present emulsion system.

Comparative studies on FA composition and oxidative stability indicated that salmon egg PC was oxidatively more

TABLE 2
Effects of Charged Components on the Oxidative Stability of Soybean Oil TAG in Casein Emulsions in the Presence of Different PC

	Charged compounds					
PC[a]	Compound	Charge	M.W.	Number of amino groups	Number of imino groups	Time for dissolved oxygen to decrease by 20% (h)
1-PA-2-LA-PC	None	—	—	—	—	12.1
+ 1,2-diAA-PC	Stearylamine	Positive	269.5	1	0	14.1
	Spermine	Positive	202.3	2	2	19.6
	Spermidine	Positive	145.2	2	1	16.7
	Putrescine	Positive	88.2	2	0	18.8
1-PA-2-AA-PC	None	—	—	—	—	8.5
+ 1,2-diAA-PC	Stearylamine	Positive	269.5	1	0	13.4
	Spermine	Positive	202.3	2	2	18.3
	Spermidine	Positive	145.2	2	1	18.3
	Putrescine	Positive	88.2	2	0	19.5
1-PA-2-DHA-PC	None	—	—	—	—	12.3
+ 1,2-diAA-PC	Stearylamine	Positive	269.5	1	0	14.4
	Spermine	Positive	202.3	2	2	23.9
	Spermidine	Positive	145.2	2	1	18.8
	Putrescine	Positive	88.2	2	0	21.9
Milk PC	None	—	—	—	—	15.8
	Dicetylphosphate	Negative	546.9	—	—	11.0
	Stearylamine	Positive	269.5	1	0	17.3
	Spermine	Positive	202.3	2	2	20.8

[a]1-PA-2-LA-PC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphate; 1,2-diAA-PC, 1,2-diarachidonoyl-*sn*-glycero-3-phosphate; 1-PA-2-AA-PC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphate; 1-PA-2-DHA-PC, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphate.

stable than soybean PC, and no significant difference in stability was apparent between chicken egg PC and salmon egg PC in liposomes, although the average degrees of unsaturation in salmon egg PC, soybean PC, and chicken egg PC were 5.97, 3.26, and 1.96 per one PC molecule, respectively (13). Furthermore, when DHA-enriched TAG were encapsulated in the liposomes, the oxidative stability of lipids in salmon egg PC liposomes was higher (13). In the PC bilayers, the nonpolar fatty acyl tails of PC are located away from the water, and the polar head groups are located in contact with the water. Therefore, it will be more difficult for a metal catalyst or free radicals in the water phase to attack the PC molecule at the interface because the PC bilayers exist in a more tightly packed conformation. The higher oxidative stability of salmon egg PC liposomes or DHA-enriched TAG encapsulated in liposomes would be due to the formation of a tight intramolecular packing arrangement of the PC. In the present emulsion system, oil droplets are surrounded by membranes composed of protein and PC. These membranes can protect TAG in the droplet interior from oxidative attack from the interface. Therefore, the conformation of PC layers at the interface also would be an important factor for the oxidative stability of soybean oil TAG in the emulsion.

Oxidation of milk lipids is an important cause of flavor deterioration of dairy products. In Japan, powdered milk for infants contains DHA and arachidonic acid as essential nutrients. These PUFA are added to cow's milk with other nutrients by homogenization. The homogenized milk is stored and then freezed-dried. Since homogenized milk contains highly unsaturated PUFA and metal catalysts, lipid oxidation occurs readily during storage and leads to the loss of essential PUFA and the development of undesirable flavors and potentially toxic components. Therefore, the present paper gives useful information on the inhibition of lipid oxidation in protein emulsions, especially in milk-related emulsion systems.

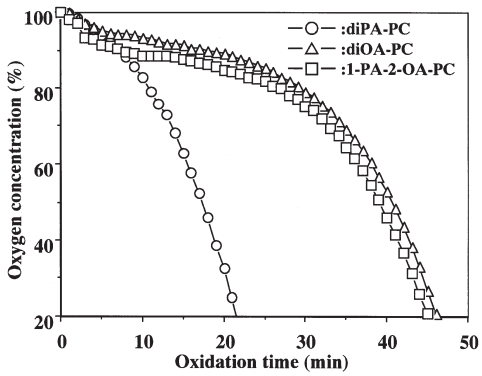

FIG. 3. Effects of PC containing palmitic acid (PA) and oleic acid (OA) on the oxidative stability of soybean oil TAG in casein emulsions. 1,2-DiPA-PC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphate; diOA-PC, 1,2-dioleoyl-*sn*-glycero-3-phosphate; 1-PA-2-OA-PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate.

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