Phospholipids plus Tocopherols Increase Soybean Oil Stability

D.H. HILDEBRAND, Department of Agronomy, University of Kentucky, Lexington, KY 40546, USA, **J. TERAO** and **M. KITO**, Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

ABSTRACT

The phospholipids (PL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purified from commercial soybean lecithin by silicic acid chromatography and preparative silica gel thin layer chromatography (TLC). Purified phosphatidylinositol (PI) was obtained commercially. Phosphatidic acid (PA) was made from PC by phospholipase D action and purified by preparative TLC. Commercial soybean tocopherols (TOC) were further purified in a florisil column. Combinations of PL and TOC were added to commercially refined, unhydrogenated soybean oil to determine the effects and interaction of PL and TOC on soybean-oil stability. Oil stability was determined by measuring the time in days of oil samples incubated at 110 C to reach a peroxide value of 100 meq/kg. Additions of TOC and all PL except PA increased the stability of the oil. PI and PE appear to be more effective than PC in increasing oil stability. The effect of the PL was not simply a matter of prooxidant metal inactivation, but rather appeared to extend the effectiveness of the TOC in free-radical termination.

INTRODUCTION

Crude soybean oil exhibits greater oxidative stability than refined oil (1-3). Part of the increased stability of soybean oil is due to the phosphatides present in crude oil (1,4). Bratkoska and Niewiadomsk (5) found that phospholipids (PL) isolated from rapeseed oil prolonged the induction period of the autoxidation of refined rapeseed oil when added at concentrations of 0-1%. The purified lecithin fraction (83.7% phosphatidylcholine [PC] and 12.7% lysophosphatidylcholine) had a greater antioxidative effect than the cephalin fraction (57.3% lysophosphatidylethanolamine, 26.1% phosphatidylserine and 16.6% phosphatidylinositol [PI]). Linow and Mieth (6) found that the hydroperoxide inhibiting effect of 0.01% α-tocopherol in methyl linoleate was increased by adding 1% PC or 1% phosphatidyethanolamine (PE). The addition of the PL also reduced the decomposition of soybean tocopherols (TOC) in linoleate oxidation. Hudson and Mahgoub (2) found that PC and PE from eggs acted as synergists with α-tocopherol in the autoxidation of lard. Among PC, PE and phosphatidic acid (PA), PE was found to be the most effective antioxidant in the autoxidation of ghee (clarified butter fat). No difference was found in the antioxidant properties of PE from sunflower *(Helianthus annus)*, peanut *(Arachis hypogea)*, soybean *(Glycine max)* or cottonseed *(Gossypium* sp.), indicating that the fatty acid portion of the molecule had no role in the antioxidant effect of PE (7).

Crude soybean oil contains 1.5-2.5% phosphatides (8). Chapman (9) found that the percentage of individual PL in the total PL of crude soybean oil ($CHCl_3/CH_3OH$, 2:1, v/v extract) from a mixture of cultivars was PA, 4.8%; PI, 20.3%; PE, 23.3%; PC, 39.0%; unknown, 12.5%. PA increases with the storage of soybeans, apparently because of endogenous phospholipase D action (10,11). Essentially all PL are removed in the degumming step of soybean oil processing except PA, which is difficult to remove (12). This study was undertaken to determine the effects of individual soybean PL on the autoxidation of soybean oil and the interaction of PL and TOC antioxidant effects.

EXPERIMENTAL PROCEDURES

Materials

PI purified from soybean seeds was purchased from Sigma Chemical Co., St. Louis, MO. Crude soybean lecithin was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Degummed and bleached and degummed, bleached and deodorized n-hexane extracted soybean oils were gifts of Ajinomoto Co., Tokyo, Japan. Crude soybean tocopherols were purchased from Eisai Co., Ltd., Tokyo, Japan. Phospholipase D was obtained from Boehringer Mannheim Gmbh, Mannheim, West Germany. Silica gel TLC plates and silicic acid were products of E. Merck, Darmstadt, West Germany. Florisil was obtained from Floridin Co., Tokyo, Japan. Pyrogalol and squalene were obtained from Nakarai Chemical Co., Kyoto, Japan. TMS-HT (hexamethyldisilazone and trimethylchlorosilane in anhydrous pyridine) were obtained from Tokyo Kasei Kogyo Co., Tokyo, Japan. All other reagents were of analytical grade.

Isolation and Purification of Phospholipids

PC and PE were isolated from commercial soybean lecithin by silicic acid column chromatography. The silicic acid (60 g) column was packed in $CHCl_3$ and the crude lecithin (1 g) in 10 mL $CHCl_3$ added to the column. The column was washed with 1 1 $CHCl_3$ and then with 1 1 $CHCl_3/CH_3OH$ (4:1, v/v). One 1 $CHCl_3/CH_3OH$ (3:2, v/v) passed through the column eluted a fraction that was largely PE. One 1 CH_3OH subsequently eluted a fraction that was largely PC. Fractions were concentrated in vacuo.

PC and PE were further purified using preparative thin layer chromatography (TLC) on Merck 2 mm silica gel plates No. 5745 with double development. $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (100:100:50:4:10, v/v/v/v/v) was used for the first development, and the plate was dried in vacuo. For the second development (in the same direction), the solvent used was $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (180:150:30:10, v/v/v/v). The PC and PE bands were identified with I_2, ninhydrin spray and molybdate reagent (13), scraped off the plates into centrifuge bottles and extracted 3 times with $CHCl_3/CH_3OH$ (2:1, v/v) in a shaking water bath at 35 C. After each extraction, the bottles were centrifuged at 1,000 g and the supernatant filtered through Whatman #2 filter paper. The PC and PE solutions were concentrated with a stream of nitrogen and the purity was further checked with TLC. The purified PC and PE yielded only one lipid spot (detected with I_2 vapor), indicating that these preparations contained no detectable lipid contaminants.

PA was made from the purified PC through phospholipase D action (14). PA was further purified using preparative TLC, as above, but using $CHCl_3/CH_3OH/NH_4$ (65:25:5, v/v/v) as the developing solvent. The PA was extracted from the silica gel and concentrated as above. The PL preparations were dried under a stream of N_2, dissolved in a specific volume of $CHCl_3$ and stored at −20 c. Quantification of PL was achieved by the procedure developed by Bartlett (15). PL was calculated as 25 times phosphorus (9).

Fatty Acid Determination

Fatty acid (FA) compositions of the degummed, bleached and deodorized oil (oil) and degummed and bleached oil (−D oil) as well as the PL were determined on a Shimadzu Gas Chromatograph (GC) 9A PF GC. The column was packed with 10% Silar 10 C on 100-200 mesh chromosorb

W. N_2 was the carrier at 45 mL/min. The temperature program was 160 to 240 C at 8 C/min. Fatty acid methyl esters were prepared by heating oil samples (ca. 1 mg) in 0.2 mL benzene and 0.8 mL sodium methoxide for 20 min at 60 C. The methyl esters were extracted with hexane containing .001% BHT.

Tocopherol Determination and Purification

One hundred mg of crude commercial soybean tocopherol mixture was dissolved in hexane and added to a column of 10 g florisil packed in hexane. The column was washed with 300 mL hexane, then with 300 mL benzene to elute the tocopherols. The benzene was removed in vacuo and the tocopherols diluted with ethanol.

Tocopherols of the oil samples were isolated as follows: 0.3 mg squalene (internal standard) was added to 1 g of the oils and the oil samples saponified by adding 4 mL 5% (w/w) pyrogalol/ethanol and placed in a boiling water bath for 2 min. One mL of 60% KOH (w/w) in water was added and samples were again placed in a boiling water bath for 2 min. The unsaponifiable matter was extracted with diethyl ether 3 times and washed with water. The diethyl ether fractions were pooled, washed again with water and dehydrated with Na_2SO_4. The ether was removed and the tocopherol preparations were dissolved in $CHCl_3$ and stored at −20 C until analyzed (less than 1 week).

Trimethylsilyl derivatives were made of the tocopherol samples by adding TMS-HT hexamethyldisilazone and trimethyl chlorosilane in anhydrous pyridine and heating at 60 C for 5 min. These samples were analyzed by GLC with squalene as the internal standard. The column was 3% SE-52 and the column was maintained at 260 C during analysis. Putative tocopherol peaks were confirmed by analysis on a GC MS-PAC 300 consisting of a Shimadzu LKB-9000 gas chromatograph mass spectrometer and an OKI TAC 4300S minicomputer using conditions described previously (6) (Table II).

Autoxidation Studies

The oxidative stability of the oil samples was measured by the induction period in days to reach a peroxide value of 100 meq/kg (IP) of oil samples incubated in 110 C.

TABLE I

Fatty Acid Composition of PL and Oils

Sample	Percentage of fatty acid				
	16:0	18:0	18:1	18:2	18:3
Oil	10.4	3.9	24.1	55.0	6.7
−D oil[a]	10.2	4.0	23.0	54.7	8.2
PC	15.5	4.4	11.9	62.5	5.7
PE	29.8	4.6	14.0	48.7	2.8
PI	33.9	7.9	6.2	46.2	5.8
PA	15.8	4.4	9.6	64.4	5.8

[a]Refined commercial soybean oil without the deodorization step.

TABLE II

Tocopherol Composition of Oils and Purified Tocopherol

Sample	Tocopherols mg/g oil or mg/mL ethanol[a]			
	α	γ	δ	Total
Oil	0.084	0.7	0.21	0.99
−D oil	0.10	0.94	0.35	1.39
Purified TOC[b]	0.22	2.03	1.83	4.08

[a]Purified TOC added to oil-PL mixtures as an ethanol solution.
[b]Tocopherols from soybeans purified on a florisil column.

Aliquots of oil were weighed, dissolved in isooctane/ethanol (1:1, v/v) and the PV measured by the procedure of Asakawa and Matsushita (17) except that 10 mL of 0.01 N HCl was added to each sample instead of 15 mL. Oil samples (1 g) were incubated in the dark in glass vials ca. 1 cm in diameter.

Two experiments were performed with different combinations of PL and TOC (Table III). The oils were transferred to the individual sample tubes in $CHCl_3$ solutions (except the −$CHCl_3$ control). PL were added as $CHCl_3$ solutions and TOC and citric acid as ethanol solutions. Solvents of the oil–PL–TOC samples were removed under a stream of N_2 and then under a vacuum. To determine the effects of $CHCl_3$ on the oxidative stability of oil samples, samples were included in which n-hexane was the only sol-

TABLE III

Design of Experiments to Determine the Effects of PL on Soybean-Oil Stability

Treatment number	Addition mg/g oil					Remarks
	PC	PE	PA	PI	TOC	
Experiment 1						
1	–	–	–	–	–	Only oil
2	–	–	–	–	–	Only −D (not deodorized)
3	–	–	–	–	–	No $CHCl_3$ added at any step
4	–	–	–	–	1.2	TOC
5	–	–	–	–	4.0	TOC
6	5.0	5.0	2.5	2.5	1.2	All PL + TOC
7	5.0	5.0	2.5	2.5	4.0	All PL + TOC
8	5.0	–	–	–	1.2	PC + TOC
9	–	5.0	–	–	1.2	PE + TOC
10	–	–	2.5	–	1.2	PA + TOC
11	–	–	–	2.5	1.2	PI + TOC
12	5.0	5.0	2.5	2.5	–	All PL alone
13	–	–	–	–	1.2	Silica gel control
14	–	–	–	–	1.2	Citric acid control
Experiment 2						
1	–	–	–	–	–	Only oil
2	–	–	–	–	5.0	TOC
3	–	5.0	–	–	5.0	PE + TOC
4	5.0	–	–	–	5.0	PC + TOC
5	5.0	5.0	–	–	5.0	PE + PC + TOC

vent that contacted the oil (treatment 3). The effects of compounds present in the silica gel of the TLC plates were determined by developing a TLC plate, without additions, with the solvent used in PA purification and removing an amount of silica gel approximating the silica gel removed in the PL purifications. The silica gel was removed and processed as for PL purifications. The silica gel extracts were added to the oil samples as $CHCl_3$ solutions as for PL. Samples containing .02% citric acid were included to determine the effects of metal chelation on oil stability.

Statistical Analysis

Three replications were made of oil samples for all treatments. The core experiment consisted of treatments 1, 4, 5, 6, 7 and 12 of experiment 1. This consisted of a factorial combination of 3 levels of tocopherols (0, 1.2 and 4 mg/g oil) and two levels of PL (0 and 5 mg/g oil PC and PE and 0 and 2.5 mg/g oil PA and PI) (Table III). This core experiment was analyzed as a 3 x 2 factorial to determine the interaction of PL and TOC on soybean-oil stability.

Multiple comparisons of pairs among the ranked treatment means were performed by computing the restricted least significant differences (LSD) (18).

TABLE IV

Results of Experiments to Determine the Effects of PL and TOC on Soybean-Oil Stability

Experiment 1		Experiment 2	
Mean IP*	Treatment number	Mean IP	Treatment number
3.65[a]	7	2.17[a]	5
3.11[b]	6	1.62[b]	3
1.60[c]	5	1.39[bc]	4
1.51[c]	12	1.14[c]	2
1.16[d]	9	0.62[d]	1
1.08[de]	11		
0.84[ef]	14		
0.83[ef]	8		
0.76[f]	2		
0.76[f]	4		
0.75[f]	1		
0.72[f]	3		
0.68[f]	13		
0.60[f]	10		

*IP = induction period in days to reach a peroxide value of 100 meq/kg. Treatment means followed by the same letter are not significantly different at the 5% level as determined by restricted LSD.

RESULTS

The fatty acid composition of the oil was similar to that of the −D oil except that the oil had a lower linolenic acid (18:3) content (Table I). As expected, PA had a very similar FA composition to PC (indicating low FA oxidation of the PA made from the PC), which had a very similar FA oxidation of the PA made from the PC), which had a very similar FA composition to the oil, except that PA and PC were much lower in oleic (18:1) and much higher in palmitic acid (16:0). PE and PI were very high in palmitic acid and PE was very low in linolenic acid. Linoleic acid (18:2) was the most abundant FA of all the lipid molecular species.

The percentages of the 3 detected tocopherol isomers—α, γ and δ—were very similar for the oil and −D oil, although as expected, the oil had less total TOC (Table II). The commercial TOC (from soybean oil-deodorization) had a higher percentage of δ and lower α and γ isomers.

A highly significant (1% level) effect of treatments and of PL, TOC and PL x TOC (interaction) (Tables III, IV) was found. The treatment of experiment 1 with all PL and the highest TOC level (treatment 7) resulted in an IP that was significantly longer than any other treatment (Table IV). All PL plus the intermediate TOC level (treatment 6) yielded an IP that was significantly greater than all treatments except treatment 7. The highest level of TOC alone (treatment 5) was not significantly different from PL alone (treatment 12) but was more effective in delaying the IP than all other treatments except 7 and 6. Five mg/g oil PE + 1.2 mg/g oil TOC, and 2.5 mg/g oil PI + 1.2 mg/g oil TOC were next in efficacy in delaying the IP. All other treatments of experiment 1, including PC + TOC, PA + TOC, 1.2 mg/g oil TOC, silica gel and citric acid controls, were not significantly different.

The results of experiment 2 corroborate those of experiment 1 (Table IV). Five mg/g oil PC and PE + 5 mg/g oil TOC was most effective in delaying the IP. The next most effective was PE + TOC, then PC + TOC and finally, TOC alone. The oil had a significantly shorter IP without any of the above additions.

DISCUSSION

The results presented here demonstrate clearly that added PE, PC and PI, alone and in combination with TOC, increased oil stability. The increased oil stability is not a result of metal chelation (treatment 14), the $CHCl_3$ used to dissolve the PL (treatment 3) or any materials extracted from the silica gel (treatment 13). This supports the results of Watts et al. (9), who found that the antioxidant effect of sodium acid pyrophosphate powder in preventing wheat flour lipid oxidation is caused by a synergism with tocopherol rather than by a special ability of the pyrophosphate to bind traces of Cu.

The results of these studies are consistent with those of Bhatia et al. (7), who found that among PC, PE and PA, PE was the most effective antioxidant in preventing the autoxidation of butter fat. Per mg of added PL, PI appears to be the most effective of the PL tested for antioxidant activity in the study reported here. The amine group of PE and PC and the reducing sugar of PI can apparently all facilitate hydrogen or electron donation to TOC. The lag phase of autoxidation lasts until a primary antioxidant such as TOC (a free-radical terminator) is exhausted, at which point the chain reaction is uninhibited, resulting in rapidly accelerating lipid oxidation. PI, PE and PC all extend the effectiveness of TOC by delaying the irreversible oxidation of TOC to tocopheryl quinone, thereby delaying the IP (4). The lack of the effectiveness of PA in improving oil stability contrasts with the results of Stuckey (4). However, Stuckey (4) used manhaden oil and the inactivation of prooxidant metals may have been a factor in that study.

The oil samples used in this study contained considerable amounts of TOC, but undetectable PL, explaining how the additions of PL alone delayed the oxidation of the oil by acting synergistically with the endogenous TOC in the oils. The strong synergistic antioxidant effect in oils of the above PL, with high surface energy, suggest an interfacial (lipid-air) action (20).

Degumming is necessary in soybean-oil refining for international trading (8) and to reduce interference with deodorization (21) and hydrogenation (22). However, to improve the oxidative stability of some soybean lipid products, readdition of PL in the form of crude soybean lecithin, perhaps in combination with other low-cost, natural antioxidants, such as protein hydrolyzates (22,23), may be beneficial.

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Synthesis of Tetrazole from α, β—Unsaturated Carbonyl Fatty Acid

JAMAL MUSTAFA, M.S. AHMAD, JR., A. RAUF and **S.M. OSMAN,*** Section of Oils and Fats, Department of Chemistry, Aligarh Muslim University, Aligarh-202001, India

ABSTRACT

Methyl 4-oxo-*trans*-2-octadecenoate (II), when treated with excess hydrazoic acid in the presence of BF_3-etherate, produced 66% methyl 5-aza-nonadec-*trans*-2-enoate (4,5-d)-tetrazole (III), 10% methyl 5-aza-nonadec-4-oxo-*trans*-2-enoate (IV) and 7% pentadecamide (V). Individual products were characterized by spectral and elemental methods.

INTRODUCTION

Five-membered diunsaturated heterocyclics with a single carbon and four nitrogen atoms are known as tetrazoles. The method of Schmidt (1) for the synthesis of tetrazole involves rearranging 1 m of ketone with 2 m of hydrazoic acid in the presence of strong acids. Previously, when simple keto fatty acids were treated with hydrazoic acid, only amides were produced (2).

The first example of the formation of tetrazole in the steroidal and triterpenoid field was reported by Barnes et al. (3). But, in the field of fatty chemistry, tetrazoles have not been reported. Tetrazoles have biological as well as nonbiological applications (4,5). Some applications include their use as propellants, dyestuffs, catalysts in polymerizations, intoxicants, sedatives and analgesics (4). They have also been used successfully to produce convulsions in the shock treatment for certain psychoses. These convulsive effects have extended their use as bird-management chemicals (5). With the discovery of these applications of tetrazoles, efforts were made for their synthesis.

EXPERIMENTAL PROCEDURE

The IR spectra were obtained with a Perkin-Elmer 621 spectrophotometer. A Backman DK-2A spectrophotometer was applied for the ultraviolet (UV) spectra. NMR were obtained with a Varian A-60 spectrophotometer. Chemical shifts were reported in relation to tetramethylsilane (TMS) in δ(ppm). The samples were run as 10% solution in $CDCl_3$. The abbreviations "s, m, d, t and br" stand for singlet, multiplet, doublet, triplet and broad. An AEI MS-902 spectrophotometer was used to record the mass spectra. Thin layer chromatography (TLC) plates were coated with silica gel G and developed with a mixture of benzene-ether-acetic acid (70:30:1, v/v). The spots were visualized by charring with a 20% aqueous solution of perchloric acid. The synthesis of (II) from (I) was carried out by the method of Nakayama et al. (6). Compound II has identical spectral and cochromatographic behavior as an authentic sample (7).

Reaction of (II) with Excess of Hydrazoic Acid: (Scheme 1)

The method of Moural and Syhora (8) was used to prepare a hydrazoic acid solution. Methyl 4-oxo-*trans*-2-octadecenoate (II) (2 g) in benzene (10 mL) was added drop by drop over a period of 4 hr to a cooled solution of hydrazoic acid and boron-trifluoride (1.5 ML, freshly distilled). The mixture was stirred for 72 hr and the solvent was removed under reduced pressure. The residue was dissolved in ether, washed with water, sodium bicarbonate (5%), water and dried over anhydrous Na_2SO_4. When the ether had evaporated, a solid (1.87 g) was obtained that was chromatographed over silica gel (40 g) to give three products. Elution with benzene:ether (96:4, v/v) gave (III). Crystallization at 10 C from petroleum ether (60-80 C) gave a yield of 66%, m.p. 52 C. (Found: C, 65.01%; H, 9.6%; N, 15.4%. Calcd. C, 65.1%; H, 9.7%; N, 15.9%.) Further elution with benzene:ether (85:15, v/v) gave (IV). The yield is 10%, m.p. 96 C. (Found: C, 69.6%; H, 10.5%; N, 4.0%. Calcd. C,

*To whom correspondence should be addressed.