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Phospholipid-Phospholipid Interaction in Soybean Oil

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ABSTRACT

Phospholipid-phospholipid interaction in soybean oil is described. Phosphatidylcholine was effectively removed from soybean oil by degumming (water hydration), whereas phosphatidylethanolamine and phosphatidic acid were hardly hydratable. However, the degree of their hydration increased in the presence of phosphatidylcholine. The spectrophotometric assay based on charge transfer interaction between 7,7,8,8-tetracyanoquinodimethane and phospholipids at 480 nm was used to determine the formation of phospholipid micelles in soybean oil. The critical micelle concentrations were 0.085, 0.84 and 2.6 mM for phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid, respectively. Phosphatidylcholine interacted with phosphatidylethanolamine or phosphatidic acid to form mixed micelles. The critical micelle concentrations of phosphatidylcholine-phosphatidylethanolamine mixture and phosphatidylcholine-phosphatidic acid mixture were 0.16 and 1.3 mM, respectively. The degree of hydration of phospholipids was related to their critical micelle concentrations. Interaction of phosphatidylcholine with phosphatidylethanolamine or phosphatidic acid was confirmed by determining the changes in the chemical shifts of ^{31}P NMR spectra.

INTRODUCTION

The soybean phospholipids in crude soybean oil which consist of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (1,2) are normally hydratable with water, i.e., they swell, form gels which then precipitate from the oil and are easily separated by centrifugation. This processing step forms the basis for manufacture of food-grade lecithin, and worldwide production is estimated at 100,000 tons (3). Water hydration normally removes about 80-95% of the phospholipids as shown by elemental phosphorus (4). The residual phospholipids are subsequently removed from soybean oil by alkali refining (5).

However, under certain conditions of bean storage, handling or processing, presumably enzymatic (2,6) processes degrade soybean phospholipids and render them nonhydratable (2,7). The nonhydratable soybean phospholipids are strong emulsifying agents and their presence during refining can entrain considerable amounts of acid in the soupstock, thereby increasing the refining loss.

In this paper, the behavior of phospholipids and phospholipid-phospholipid interaction in soybean oil are studied by ^{31}P NMR spectroscopy, determination of critical micelle concentration (cmc) and the way they respond to the degumming method.

EXPERIMENTAL PROCEDURES

Materials

CM52 carboxy cellulose was used (Whatman Ltd., Springfield, England). Alumina Woelm N-Super I was from Woelm Pharma GmbH & Co., Eschwege, West Germany. Thin layer plates were products of E. Merck (Art 5721), Darmstadt, West Germany. Phospholipase D was obtained from Boehringer Mannheim Gmbh, Mannheim, West Germany, and 7,7,8,8-tetracyanoquinodimethane (TCNQ) was purchased from Aldrich Chemical Co., Milwaukee, WI. All other reagents were analytical grade.

Isolation and Purification of Phospholipids

PC and PE were isolated from commercial soybean lecithin by alumina column chromatography (8). The PE fraction was further purified by carboxy methyl cellulose column chromatography according to Comfurius and Zwaal (9). Phosphatidic acid was prepared from PC by phospholipase D treatment (10). The phosphatidic acid preparation contained Ca^{2+} (mol ratio of phosphatidic acid/Ca^{2+} = 2). The purity of the phospholipid preparations was checked by thin layer chromatography (TLC) (11).

TABLE I

Hydration of Phospholipids in Oil

Degumming	Phospholipids in oil (μg/g oil)				
	PC[a]	PA	PE	PA+PC	PE+PC
Before	1,700	2,000	1,900	1,900+2,490	2,100+2,100
After	trace	1,300	470	770+1,080	trace+trace

[a]PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine.

Phospholipid Dissolved in Soybean Oil

Phospholipids were dissolved in soybean oil by the following procedures: *n*-hexane solution of soybean oil (0.1 g/mL), which had been extracted with *n*-hexane from soybeans and completely degummed with citric acid (prepared by Oil and Fat Institute of Ajinomoto Co.), and a chloroform solution of phospholipids (10 mg/mL) were mixed, and the solvents were then removed in vacuo.

Hydration of Phospholipids in Soybean Oil

Phospholipids were hydrated and removed from soybean oil according to List et al. (12). Soybean oil containing phospholipids (3 g) was incubated at 60 C for 5 min under nitrogen, after which 7.5 μL of acetic anhydride was added and the mixture shaken vigorously for 3 min. The mixture was then vigorously shaken with 60 μL of 0.1 M EDTA for another 3 min. After centrifugation, phospholipids remaining in the soybean oil were determined by measuring phosphorus content according to Bartlett (13), followed by column chromatography (9) and separation by TLC with the solvent system $CHCl_3/CH_3OH/CH_3COOH$ (65:25:8, v/v/v).

Determination of cmc Using TCNQ Solubilized Technique

Aliquots of phospholipid were dissolved in soybean oil as described before, and then by dilution with soybean oil, a series of samples containing different concentrations of phospholipids were obtained. About 1.5 mg of TCNQ was added to 1.5 g of the oil containing the phospholipids, and the mixture was vigorously shaken for 5 hr at room temperature (21±2 C). After sedimentation of excess TCNQ by centrifugation at 800 × g for 20 min, the absorption spectra of the solubilized TCNQ were measured. The cmc was taken as the break in the curve between the absorbance at 480 nm or 850 nm and the logarithm of phospholipid concentrations. The absorption spectra were measured with Shimadzu MPS-500 spectrophotometer. Measurements of absorbances at 480 and 850 nm were made in a Carl Zeiss PMQ spectrophotometer. Soybean oil was used as a reference.

Measurements of ^{31}P NMR Spectra

^{31}P NMR specta with wide band proton decoupling (decoupling frequency 15,400 Hz) were recorded at 36.4 MHz on a Hitachi FTNMR spectrometer type R-900 at 35 C. The chemical shifts were measured using 85% H_3PO_4 as an external reference. Accumulated free induction decays were obtained from 3,000 to 65,000 transients employing a 40° pulse width and 1-sec interpulse time.

RESULTS

Hydration of Phospholipids in Soybean Oil

Phospholipids dissolved in soybean oil were degummed by hydration. PC was completely removed from the oil, but phosphatidic acid was highly nonhydratable (Table I). However, about 60% of phosphatidic acid was removed in the presence of PC. About 25% of PE remained in the oil after the degumming treatment (Table I). This, too, was removed to a great extent in the presence of PC. Thus, it appears that phosphatidic acid or PE became more hydratable in the oil due to their interaction with PC than by themselves.

Absorption Spectra of TCNQ Solubilized in Soybean Oil Containing Phospholipids

The TCNQ solubilization technique is useful for determining cmc of oil-soluble surfactants in nonaqueous media (14,15). This technique was applied to examine micelle formation of phospholipids in soybean oil.

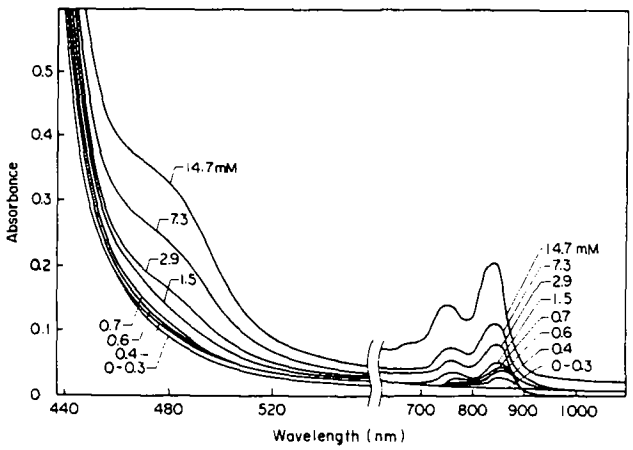

FIG. 1. Absorption spectra of TCNQ solubilized in soybean oil containing phosphatidic acid. The numbers indicate the concentration of phosphatidic acid as mmol/kg oil.

The absorption spectra of TCNQ solubilized in soybean oil containing phosphatidic acid is shown in Figure 1. Characteristic absorption bands were observed with the increase in phosphatidic acid concentration. The absorbance at 480 nm suggested the charge transfer interaction between TCNQ and phosphatidic acid (14). Absorbances at 750 and 850 nm were those of TCNQ anion radicals which resulted from the charge transfer interaction (14). The spectra analysis, therefore, indicates the formation of a phosphatidic acid micelle in soybean oil.

The similar variations in the spectra of TCNQ were found in soybean oil containing PC, PE, PC-PE mixture or PC-phosphatidic acid mixture (data not shown).

Determination of cmc of Phospholipids in Soybean Oil

The absorbance at 480 nm of TCNQ solubilized in soybean oil containing phospholipids was plotted as a function of the logarithm of phospholipid molarity (mmol/kg oil). The cmc was taken as the break in the curve between absorbances at 480 nm and the logarithm of phospholipid concentrations (Fig. 2). The cmc of PC, PE and phosphatidic acid were 0.085, 0.84 and 2.6 mM, respectively (Fig. 2, top). The cmc of PE or phosphatidic acid was changed in the presence of PC. It seems likely that interaction of PC with PE or phosphatidic acid in soybean oil occurred, and that they formed mixed micelles. The cmc of the mixed micelles were intermediate values between cmc of individual phospholipids constituting the mixed micelles (0.16 mM for the PC-PE mixture, 1.3 mM for the PC-phosphatidic acid mixture) (Fig. 2, bottom).

Plots of TCNQ absorbance at 850 nm gave results similar to those already described (data not shown).

^{31}P NMR Spectra of Phospholipids in Soybean Oil

A ^{31}P NMR spectrum of PC in soybean oil (2.7 mM) resulted in a broad peak with a chemical shift at -12.71 ppm (Fig. 3). Phosphatidic acid (2.9 mM) gave a sharp peak at -0.92 ppm. However, when PC and phosphatidic acid were present together, 2 sharp peaks at -1.42 and -2.90 ppm were obtained, and these differed from the respective chemical shifts of PC and phosphatidic acid (Fig. 3). PE (2.7 mM) showed a sharp peak with a chemical shift at -0.50 ppm (Fig. 4). In the presence of PC, a broad peak appeared at +4.27 ppm with the concomitant disappearance of the 2 peaks at -12.71 and -0.50 ppm (Fig. 4). The areas of those spectra obtained at the same concentration were

the same. These results suggest that PC thoroughly interacted with PE or phosphatidic acid in soybean oil.

DISCUSSION

The formation of micelles of some surfactants in nonaqueous solvent is affected by a trace amount of water existing in the solvent or air (16). There is a possibility that a trace amount of water was present in the soybean oil used, since the oil could not be completely protected from exposure to air during the experiments. It is ambiguous whether phospholipids require water for the formation of micelles in the oil. However, it was noted that the degree of hydration (extent of degumming) of phospholipids was related to their cmc. The lower the cmc, the higher the hydration.

The ^{31}P NMR spectra of phospholipids dispersed in aqueous media have been studied (17,18). This is the first report regarding the ^{31}P NMR spectra of phospholipids in a nonaqueous medium such as soybean oil. The ^{31}P NMR spectrum of PC resulted in a broad peak, whereas those of PE and phosphatidic acid showed sharp peaks (Figs. 3 and 4). The size, shape and overall motion of phospholipid dispersions in aqueous media significantly influence the line shapes of ^{31}P NMR spectra (15,19,20). The line shapes of unilamellar phospholipid vesicles become increasingly broader with the increase in their size (20). Therefore, the broad peak of PC, compared to those of PE and phosphatidic acid, may be due to the formation of larger micelles with higher aggregation numbers. On the other hand, the sharp peaks of phosphatidic acid and PE may reflect the

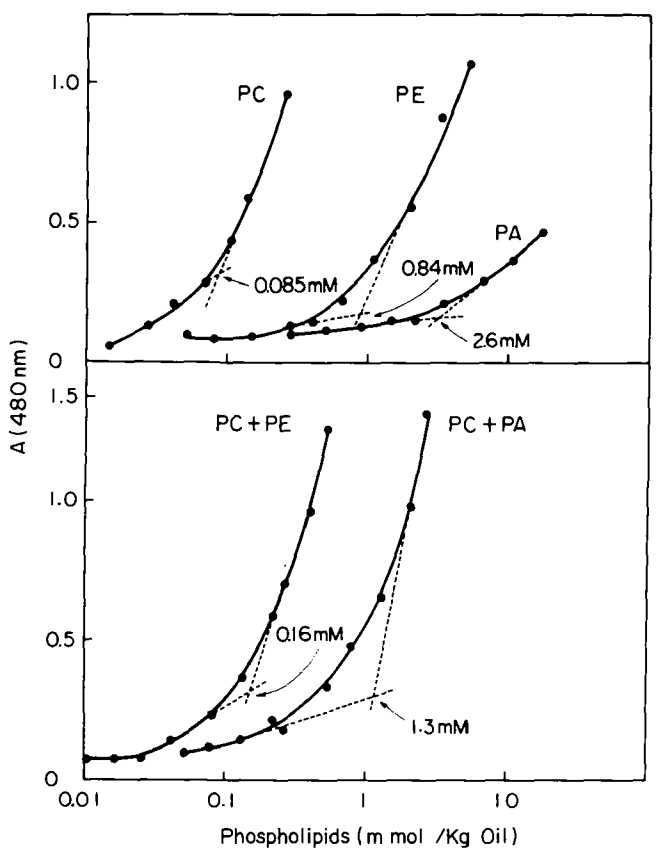

FIG. 2. Plots of absorbances of TCNQ solubilized in soybean oil containing phospholipids as a function of logarithm of phospholipid molarity. The numbers indicate cmc. PC+PA=soybean oil containing equimolar mixture of PC and PA; PC+PE=soybean oil containing equimolar mixture of PC and PE. Abbreviations are the same as those in Table I.

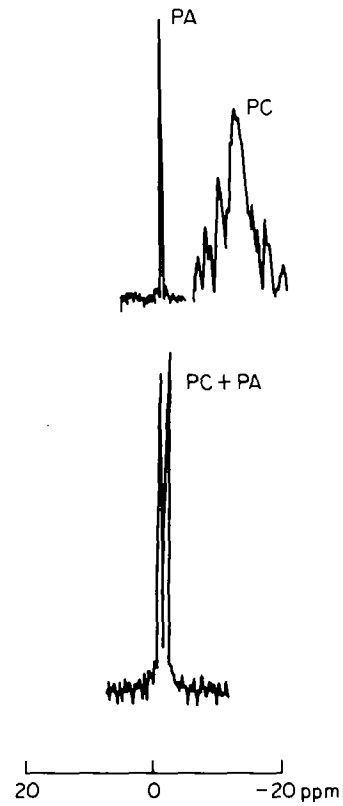

FIG. 3. 36.4 MHz ^{31}P NMR spectra of phospholipids in soybean oil. PC=2 mg/g oil; PA=2 mg/g oil; PC+PA=2 mg each/g oil. The heights of spectra of PA and PC+PA were drawn at half scale. Abbreviations are the same as those in Table I.

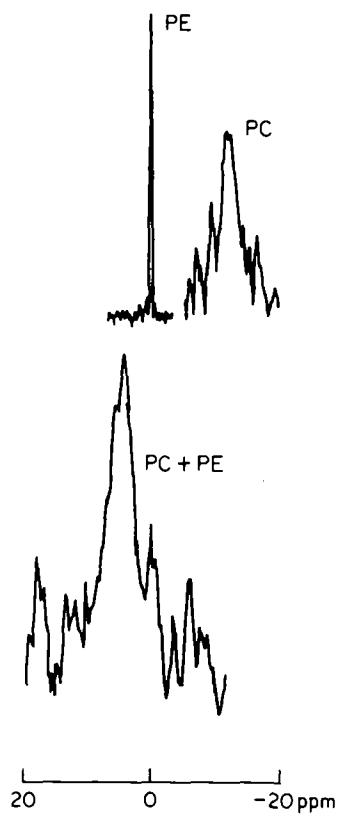

FIG. 4. 36.4 MHz ^{31}P NMR spectra of phospholipids in soybean oil. PC=2 mg/g oil; PE=2 mg/g oil; PC+PE=2 mg each/g oil. The height of spectrum of PE was drawn at half scale. Abbreviations are the same as those in Table I.

presence of smaller micelles with lower aggregation numbers. The chemical shifts of respective phospholipids were changed when 2 phospholipid classes were simultaneously present. The PC-PE mixture showed a broad peak, suggesting the formation of larger micelles with higher aggregation numbers. Two sharp peaks appeared in the simultaneous presence of PC and phosphatidic acid. When PC and phosphatidic acid were present, TCNQ was gradually solubilized with the increase in phospholipid concentration (Fig. 2, bottom). Therefore, the micelles with different aggregation numbers are assumed to be formed by steps into a definite association, as in the case of surfactants (21,22). Thus the 2 peaks in the ^{31}P NMR spectrum may reflect 2 species of small aggregates with lower aggregation numbers. These results indicate that PC may interact with PE or phosphatidic acid.

The simultaneous presence of 2 phospholipid classes changed their respective cmc (Fig. 2), suggesting that mixed micelles of PC with PE or phosphatidic acid were formed due to their interaction. The phospholipid-phospholipid interaction suggested by the results of hydration (Table I) and cmc (Fig. 2) experiments is supported by the results from ^{31}P NMR.

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Volatile Sulfur Compounds and Other Headspace Constituents of North Sea Fish Oils

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ABSTRACT

Headspace fractions of industrial oils produced from North Sea fish have been studied with emphasis on their sulfur-containing constituents, suspected inhibitors in the subsequent, catalytic oil hardening process. Fourteen individual, volatile sulfur compounds have been identified, accounting for virtually the total amount of such compounds in the volatile fraction. Sulfides, linear and cyclic di- and polysulfides, and a homologous series of methyl thiolesters, together make up the chemical patterns. Gas chromatography combined with mass spectrometry revealed a complex, but surprisingly constant pattern of the total, volatile fractions of several North Sea fish oils. About 100 individual compounds, including those containing sulfur, have been fully or partly identified. The structural characteristics and possible origin of the various types of compounds are discussed briefly.

INTRODUCTION

Fish oils are evaluated industrially by, among other things, their readiness to undergo catalytic hydrogenation (hardening), a process subject to marked inhibition by various factors. Among these, the sulfur content obviously plays a preeminent role (1,2). Several studies have indicated that certain water-soluble sulfur compounds, notably cysteine and methionine, rapidly undergo degradation to simple sulfur-containing products through bacterial activity in spoiling fish, whereas autolysis seems to be of minor importance in this connection (3,4). Despite the proven production under these circumstances of a few sulfur volatiles such as hydrogen sulfide, methanethiol and dimethyl sulfide, little is known about sulfur compounds present in industrial fish oils. On the assumption that insight into the chemical nature of the fat-soluble sulfur compounds present in or produced during the processing of spoiling fish may prove helpful in their removal or inactivation as inhibitors in the industrial hardening process, a systematic study of such compounds was undertaken in our laboratory. This paper reports the identification of the individual sulfur compounds encountered in the *volatile* fractions of typical, unrefined fish oils of North Sea origin. The studies were conducted by subjecting the oil headspaces to gas chromatographic (GC) analysis with simultaneous recording of both the total pattern of volatiles and, selectively, the sulfur-containing-constituents, supplemented by gas chromatographic/mass spectrometric (GC/MS) analysis. Though of less importance in the present context, an array of sulfur-free fish oil volatiles were encountered during our studies. They are discussed briefly at the end of this paper.

MATERIALS AND METHODS

All oils studied in this work were of industrial origin,