Preparation and Surface- Active Properties of Hydroxy and Epoxy Fatty Acid-Containing Soy Phospholipids

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ABSTRACT: This paper deals with the ester-ester interchange reaction using an immobilized lipase (Lipozyme TL IM) as biocatalyst and sodium methoxide as a chemical catalyst for incorporating oxygenated FA such as 12-hydroxyoleic , 12-hydroxystearic, and 12-epoxyoleic in soy phospholipids. As much as 65% hydroxyoleic acid, 57% hydroxystearic acid, and 43% epoxyoleic acid were incorporated in soy phospholipid with lipase. The corresponding incorporations of these acids were 51, 49, and 35%, respectively, by using the chemical catalyst. Interface properties such as critical micelle concentration (CMC), effectiveness of interfacial tension reduction (γ_{CMC}), maximum surface excess concentration (Γ_{max}), and minimum area/molecule at the interface (A_{min}) of the modified products were measured and found to be different from those of the original soy phospholipids.

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KEY WORDS: Critical micelle concentration, epoxy fatty acids, hydroxy fatty acids, lipase, minimum area/molecule, surface excess concentration, surface property.

Phospholipids have distinct surface-active properties for which they are used in the food, feed, cosmetics, and pharmaceutical industries (1–3). These properties of phospholipids can be modified by enzyme-catalyzed changes in their FA composition and can lead to new applications as emulsifiers. For example, Brockerhoff *et al.* (4) reported a lipase-catalyzed regiospecific 1-position transesterification of FA in phosphatidylcholine and phosphatidylethanolamine. They used *Rhizopus delemar* lipase in buffer with phosphatidylcholine and oleic acid as substrate. Yoshimoto *et al*. (5) used polyethylene glycol-modified *Candida cylindracea* lipase in benzene to incorporate PUFA into phosphatidylcholine (5). For transesterification of phosphatidylcholine and phosphatidylethanolamine with different FA, Yagi *et al.* (6) used a two-phase water system. Yoichiro and Setsuki (7) also used a water/oil system but used sardine oil as source of PUFA for incorporation into soy phospholipid (7). Mutua and Akoh (8) successively incorporated n-3 PUFA in the phospholipid moiety with *Mucor miehei* lipase. Ghosh and Bhattacharyya (9) incorporated capric, lauric, and myristic acids into soy phospholipids at the *sn*-1 position and examined their surface properties. Ghosh, Das, and Bhattacharyya (10) also reported the incorporation of hydroxy and epoxy FA in soy phospholipids using *Mucor miehei* lipase in an ester-ester interchange reaction lasting 6 h. The incorporation of ricinoleic acid into phosphatidylcholine isolated from soybean and egg lecithin was achieved using phospholipase A_1 (20% based on phosphatidylcholine); a 1:5 mole ratio of phosphatidylcholine to methyl ricinoleate at a reaction temperature of 50°C for 24 h (11). The overall yield of the modified phosphatidylcholine was 35%.

The present study investigates the preparation of soy phospholipids containing oxygenated FA—including hydroxyoleic acid, hydroxystearic acid, and epoxyoleic acid (vernoleic acid)—by interesterification using an *sn*-1,3 specific lipase as biocatalyst or sodium methoxide as chemical catalyst. The surface-active properties of the resultant products were then compared with those of the original soy phospholipid.

EXPERIMENTAL PROCEDURES

Materials and catalysts. Crude soy phospholipid, supplied by Vippy Industries Ltd. (Dewas. Madhya Pradesh, India) was deoiled by repeated extraction with cold acetone. Finally the deoiled phospholipid was isolated by removing residual solvent under vacuum on a rotary evaporator and stored in a nitrogen environment (8).

The catalytic immobilized lipase (Lipozyme TL IM, from *Thermomyces lanuginosa*) was provided by Novo A/S (Copenhagen, Denmark).

The NaOMe (20% NaOMe in methanol) used as chemical catalyst was prepared in the laboratory by reacting an exact amount of metallic sodium in anhydrous methanol.

The methyl esters of 12-hydroxyoleic acid from castor oil, 12-hydroxystearic acid from hydrogenated castor oil, and 12-epoxyoleic acid (vernolic acid) from *Vernonia anthelmintica* oil (Somraj oil) were used.

All the solvents were supplied by S.D. Fine Chemicals Ltd. (Mumbai, India).

Ester interchange reaction. (i) By enzyme catalyst. De-oiled soy phospholipid and the methyl esters of individual hydroxy and epoxy FA were added in a 1:5 (w/w) ratio to a 50-mL round-bottomed flask fitted with a water condenser and stirred by a magnetic stirrer. Chloroform (15 mL) was added to solubilize both the phospholipids and oxygenated FA; this solvent was chosen in preference to a hydrophobic solvent such as *n*-hexane in which the solubility of the hydroxy acids is low. Toluene is not used because its b.p. is much higher than that of chloroform. The temperature was kept at 55–60°C instead of keeping either at 55°C or 60°C, and the reaction continued for

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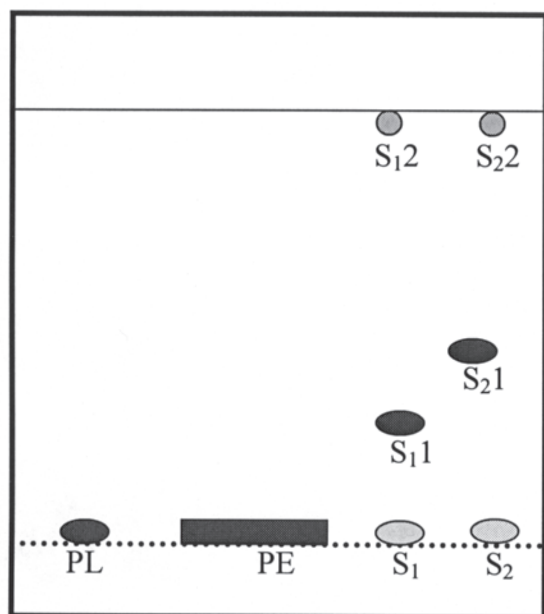

FIG. 1. TLC separation of enzymatically modified soy phospholipids isolated as acetone-insoluble portion. Solvent system: 80:20 (hexane/diethyl ether); PL, original soy phospholipids; PE, enzymatically modified soy phospholipids (acetone-insoluble); PC, chemically modified soy phospholipids (acetone-insoluble); S_1, methyl ester of hydroxy FA; S_2, methyl ester of epoxy FA; $S_1$1, oxygenated FA (hydroxyl ester); $S_2$1, oxygenated FA (epoxy ester); $S_1$2 and $S_2$2, methyl esters of nonoxygenated FA.

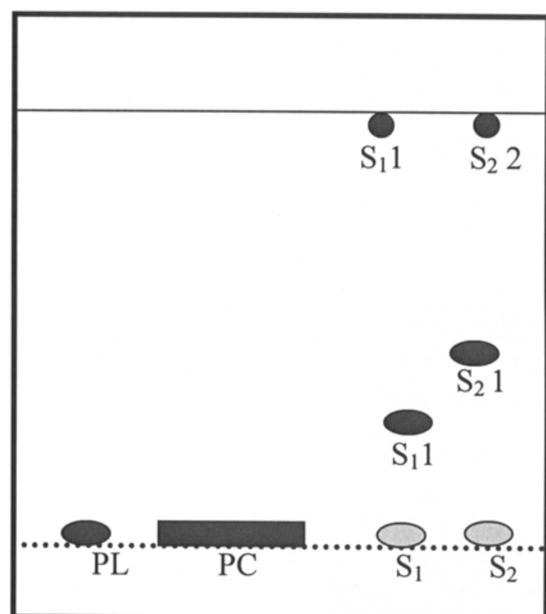

FIG. 2. TLC separation of chemically modified soy phospholipids isolated as acetone-insoluble portion. For abbreviations see Figure 1.

7 d with 10% (by weight of the reactants) of TL IM lipase (the lipase initially contained 2% water). After the reaction, the product mixture first was filtered to remove the enzyme, and then the solvent was removed under vacuum. After complete removal of chloroform the transesterified phospholipid was separated from both the unreacted methyl esters of oxygenated FA and the FA replaced from soy phospholipid by several extractions with acetone in which the methyl esters were soluble. The acetone-insoluble portion contained the transesterified soy phospholipids. The purity of the modified phospholipids was tested further by preparative silica gel TLC; no other lipid components appeared on the chromatogram as evident from Figure 1. The reaction was monitored by sample withdrawal on a daily basis until there was maximal incorporation of the hydroxy and epoxy FA.

(ii) By chemical catalyst. A homogeneous mixture of deoiled phospholipid and methyl ester of each oxygenated fatty (1:2 weight ratio) was interesterified for 30 min by vigorously stirring at 75–80°C temperature with 0.4% (by weight of the reactants) NaOMe (20% NaOMe in methanol) as catalyst. After the reaction, an appropriate volume of 1 N HCl was added to acidify the mixture to pH 3 to destroy the catalyst and decompose the soaps formed in the case of the hydroxy acid-containing products. In the case of epoxy acid-containing products, no acidification was done in order to protect the epoxy group in the product molecule. To each product, 30 mL of chloroform was added. Additionally, a sufficient amount of anhydrous sodium sulfate was added to remove water from the acid treatment and to remove soap in case of the epoxy product. The chloroform solution was then filtered to remove sodium sulfate and soap materials. Next, chloroform was removed completely under vacuum to yield the total reaction mass. The transesterified phospholipid product was isolated from the mixed methyl esters of the unreacted oxygenated FA and the replaced FA by several extractions with acetone as already described. Again, the acetone-insoluble portion contained only the modified phospholipids and no other lipid components. The modified phospholipids were examined as before by TLC for their purity (Fig. 2), and their FA compositions were determined by a combination of TLC and GLC.

Chemical characterization of the modified phospholipids: The determination of the compositions of the FA of the original and modified phospholipids were made by the TLC and GLC. A small amount of each modified phospholipid (i.e., the acetone-insoluble portion) was dissolved in chloroform, and 50 μL of this chloroform solution was applied as a band on the TLC plate (silica gel G, 0.2 mm). The TLC plate was developed with a solvent system of hexane/diethyl ether (90:10 vol/vol) to separate the modified phospholipids from the methyl esters of hydroxy FA (and any other FA remaining in trace quantities after the acetone extraction). Lipid bands separated on the TLC plate were visualized by iodine vapor, and the band remaining on the point of application was extracted by Folch solvent (methanol/chloroform 2:1) system several times to obtain the purified modified phospholipid products.

FA analysis of modified phospholipids. The solvent was removed by evaporation under vacuum to recover the modified phospholipid. The recovered product was dissolved by 1 mL diethyl ether. Then 1 mL of 0.5 N methanolic KOH solution was added, and the mixture was shaken for 10 min. Next, 1 N

HCl (1 mL) was added to acidify. The FAME were taken up in petroleum ether (b.p. 40–60°C) (12). For epoxy acid-containing products, the FAME were taken up in petroleum ether and water-washed repetitively until the water washings were neutral. The methyl esters were analyzed by GLC.

GLC. The FAME were analyzed by GLC on a Hewlett-Packard gas chromatograph (HP 5890A; Palo Alto, CA) equipped with an FID. The analysis was done using a Chromosorb-WHP coated with 10% diethylene glycol succinate [DEGS: 6′ × 1/8″ i.d. (183 cm × 3 mm)]. The column temperature was maintained at 190°C, and N_2 was used as a carrier gas at a flow rate of 30 mL/min. The different FAME and the standard samples were separated under the same conditions.

Measurement of interfacial tension. The interfacial tension of solvent (chloroform) and of chloroform solutions of the original and modified phospholipids against water was determined at 27°C by the drop-weight method (13) with an Agla Micrometer Syringe (Burroughs, Wellcome & Co., London, England) based on the equation

$$\gamma_{1/11} = (\rho_1 - \rho_{11})\nu\, g/2\pi rf \quad [1]$$

where γ = interfacial tension at the liquid 1 and 11 interface, ν = volume of the drop of the liquid, g = gravitational acceleration, r = radius of the capillary (<1 mm), and f = correction factor. The measurement was done in a closed system to avoid evaporation loss. From the experimental determination of the volume corresponding to 10 drops, the volume per drop (ν) and $r/\nu^{1/3}$ were calculated. The correction factor corresponding to $r/\nu^{1/3}$ was finally obtained from the values determined and tabulated by Harkins and Brown (13). The critical micelle concentration (CMC) values of the original soy phospholipids and the enzymatically and chemically modified products were derived from the break of interfacial tension vs. molar concentration (log C) graph.

RESULTS AND DISCUSSION

In the present study the methyl esters of 12-hydroxyoleic acid (ricinoleic) from castor oil, 12-hydroxystearic acid, and 12-epoxyoleic acid (vernolic acid) were used as monoester for ester-ester interchange reaction with soy phospholipids consisting of phosphatidylcholine, phosphatidic acid, phosphatidylethanolamine, and others. The interesterification reaction was done by enzymatic and chemical methodologies. The FA compositions of the oxygenated FA, the original soy phospholipids, and the modified phospholipids after incorporation of the oxygenated FA by enzymatic and chemical routes are shown in Table 1. The extent of incorporation of 12-hydroxyoleic acid, 12-hydroxystearic acid and 12-epoxyoleic acid was 65, 57, and 43%, respectively in soy phospholipid with TL IM lipase. The corresponding incorporations of the said acids were 51, 49, and 35%, respectively, using sodium methoxide as a chemical catalyst. The percentage incorporation of 12-hydroxyoleic acid is more than that of the 12-hydroxystearic acid or 12-epoxy oleic acid irrespective of the catalyst. The incorporation of the oxygenated acids (hydroxyoleic acid, hydroxystearic acid, and epoxyoleic acid) was higher in the enzyme-catalyzed process than the chemical-catalyzed process. The distributions at the *sn*-1 position of the total saturated and unsaturated FA in soy phospholipids before reaction were about 15 and 33.7%, respectively, as determined by the phospholipase A_2 hydrolysis reaction. On the basis of the distribution pattern of FA of the soy phospholipids, the incorporation of the hydroxy acids and epoxy acid would be around 50% in the normal course of the reactions. However, for the hydroxy acids, the incorporations were much more. The distribution pattern of FA in the original soy phospholipids was examined but not in the transesterified or modified products. The much higher incorporation of oxygenated FA in the case of enzymatic process could be attributed to the intramolecular migration of

TABLE 1
FA Compositions of Original Soy Phospholipid, Individual Hydroxy and Epoxy FA Esters, and the Transesterified Phospholipids by Enzyme and Chemical-catalyzed Reactions[a]

	Composition of FA (%w/w)								
Sample	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	C_{MEPo}	C_{MHSA}	C_{MHO}	Other
MHO	2.1	1.6	7.5	9.2	—	—	—	77.8	3.0
MHSA	1.1	—	11.5	—	—	—	87	—	0.5
MEPO	4.1	1.5	3.5	12.4	—	78.2	—	—	—
Soy phospholipid	27.3	1.6	14.7	52.5	3.8	—	—	—	0.1
P2e	6.4	1.4	7.1	17.4	1.2	—	—	65.1	1.4
P3e	7.0	2.2	6.7	23.0	3.2	—	57.6	—	0.7
P6e	8.2	2.0	7.3	35.5	2.5	43.1	—	—	1.4
P2C	13.4	0.7	7.9	23.0	1.6	—	—	51.3	0.7
P3C	12.9	1.18	9.2	25.2	1.8	—	49.2	—	0.7
P6C	14.2	2.0	9.5	35.5	2.5	35.1	—	—	1.4

[a]MHO , methyl esters of hydroxyoleic acid (12-hydroxy $C_{18:1}$); MHSA, methyl esters of hydroxystearic acid (12-hydroxy $C_{18:0}$); MEPO, methyl esters of epoxyoleic acid (12-epoxy $C_{18:1}$); P2e, phospholipid enzymatically modified by hydroxyoleic acid; P3e, phospholipid enzymatically modified by hydroxystearic acid; P6e, phospholipid enzymatically modified by epoxyoleic acid; P2C, phospholipid chemically modified by hydroxyoleic acid; P3C, phospholipid chemically modified by hydroxy stearic acid; P6C, phospholipid chemically modified by epoxyoleic acid.

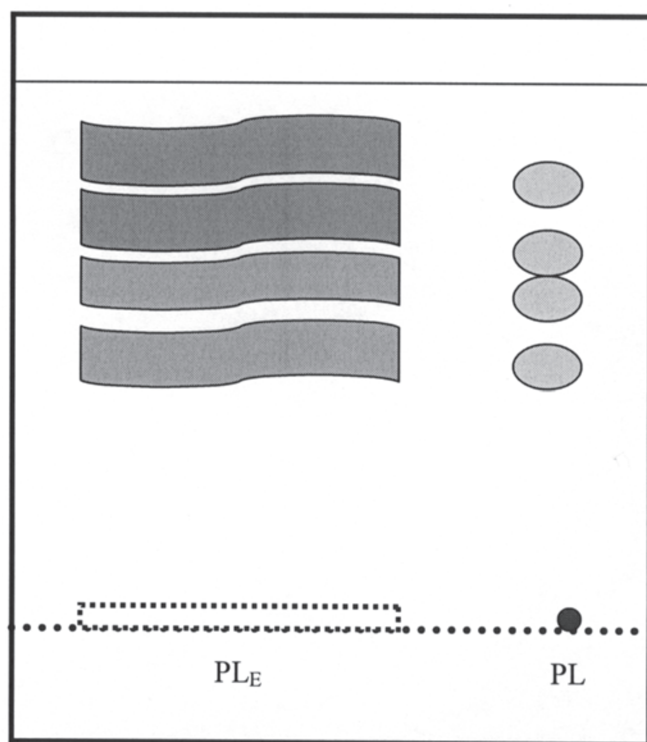

FIG. 3. TLC separation pattern on silica gel G layer of enzymatically modified phospholipids. PL$_E$, soy phospholipids enzymatically modified; PL, original soy phospholipids. Solvent system: chloroform/methanol/water/acetic acid (25:15:4:2). Staining method: iodine vapor.

the FA as indicated by the relative proportions of the 18:2 and 16:2 FA before and after reaction of phospholipids in a batch reaction for a period of 7 d in presence of the lipase (14). Perhaps TL IM behaves like a nonspecific lipase, thereby favoring more incorporation of the oxygenated FA.

The use of a chemical catalyst led to the incorporation of a lower amount of oxygenated FA, presumably owing to a lower extent of intramolecular migration of FA, or perhaps the formation of lysophospholipids was less when a chemical catalyst was used.

The modified soy phospholipids containing oxygenated FA such as hydroxy and epoxy FA were examined for their surface properties. Phospholipid distribution was not measured before and after reaction. Lysophospholipids are usually formed during interesterification reaction which is itself could make the product more hydrophilic. In the present investigation the content of lysophospholipids did not appear to be significant in the products as evidenced by TLC (Fig. 3). The extent of influence on the surface-active properties of the modified phospholipids due to the lysophospholipids formation could not be correlated. It could be that the lysophospholipids were reacylated by the lipase, as described by Haraldsson and Thorarensen (15), during the long reaction period (7 d). As a result, the amount of lysophospholipids in the products was not great enough to exert much influence in the surface-active properties.

The values of surface-active and thermodynamic properties of phospholipids in this study are tabulated in Table 2. The interfacial tension of a chloroform solution of the original and modified soy phospholipids against water was measured at 27°C. The interfacial tension of chloroform/water at 27°C was 33.5 mN/m. In presence of 0.1% soy phospholipids its value fell to 13.2 mN/m, giving a surface pressure $\pi = (\gamma_0 - \gamma)$ of 20.3 mN/m. At a similar concentration level (0.1%), the surface pressure declined to 9.9 mN/m for soy phospholipids with 65.1% 12-hydroxyoleic acid incorporated by enzymatic modification and to 15.9 mN/m with 51.3% 12-hydroxyoleic acid incorporated by chemical modification. For 12-hydroxystearic acid incorporated into soy phospholipid, the surface pressure reduction values were 14.5 mN/m for enzymatically modified product and 16.7 mN/m for chemically modified product. The same trend was observed for epoxy acid-incorporated product. The values were 12 mN/m and 15.1 mN/m for enzymatically and chemically modified soy phospholipids. The hydrophilicity increased owing to incorporation of oxygenated FA (12-hydroxyoleic acid, 12-hydroxystearic acid, and epoxyoleic acid). The FA chain lengths also increased in the modified phospholipids. These two factors combined to disperse the modified phospholipids from the chloroform/water interface to the solution side. So the interfacial tension values of enzymatically and chemically modified phospholipids increased from the original soy phospholipids. The increase of interfacial tension depends on the percentage of hydroxy or epoxy acids in soy phospholipids relative to the other FA and their positional distribution. In enzymatic or chemical processes, oxygenated FA are expected to be incorporated at both the *sn*-1 and *sn*-2 positions of

TABLE 2
Surface and Thermodynamic Properties of Original and Enzymatically and Chemically Modified Soy Phospholipid[a]

Sample	γ (dyn/cm) (0.1% chloroform solution)	γ_{CMC} (mN/m)	$\Gamma_{min} \times 10^{-10}$ (mol/cm^2)	A_{min} (Å)^2	CMC (mol/L)	ΔG_{mic} (kJ/mol)
Soy phospholipids	13.2 ± 0.08	23.6 ± 0.2	1.6 ± 0.1	103.7 ± 0.1	$(5.16 \pm 0.2) \times 10^{-5}$	24.4 ± 0.2
P2e	23.6 ± 0.08	28.4 ± 0.2	0.67 ± 0.1	244.5 ± 0.1	$(1.6 \pm 0.2) \times 10^{-5}$	27.5 ± 0.2
P3e	19 ± 0.08	24.8 ± 0.2	0.52 ± 0.1	315 ± 0.1	$(1.4 \pm 0.2) \times 10^{-5}$	27.8 ± 0.2
P6e	21.5 ± 0.08	28.2 ± 0.2	0.80 ± 0.1	207.2 ± 0.1	$(1.18 \pm 0.2) \times 10^{-5}$	28.4 ± 0.2
P2c	17.6 ± 0.08	28.3 ± 0.2	2.07 ± 0.1	80.2 ± 0.1	$(5.01 \pm 0.2) \times 10^{-5}$	24.6 ± 0.2
P3c	16.8 ± 0.08	28.5 ± 0.2	1.48 ± 0.1	112.1 ± 0.1	$(4.9 \pm 0.2) \times 10^{-5}$	24.7 ± 0.2
P6c	18.4 ± 0.08	27.8 ± 0.2	1.2 ± 0.1	138.3 ± 0.1	$(5.03 \pm 0.2) \times 10^{-5}$	24.6 ± 0.2

[a]CMC, critical micelle concentration; γ, interfacial tension at chloroform-water interface; γ_{CMC}, interfacial tension at CMC; Γ_{min}, surface excess concentration of surface-active molecule; A_{min}, minimum area/ molecule, $-\Delta G^0_{mic}$, free energy change of micellization. For other abbreviations see Table 1.

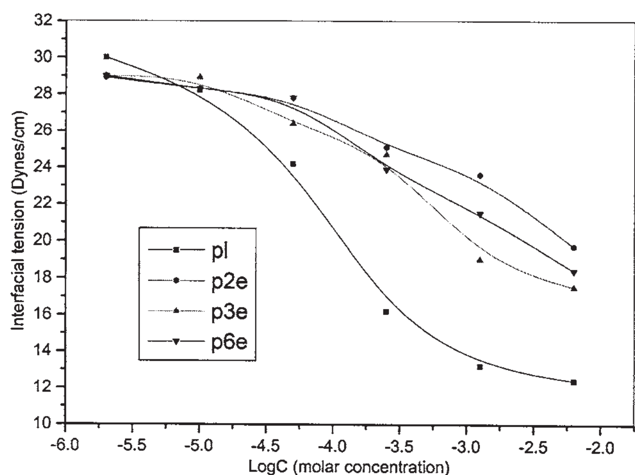

FIG. 4. Plot of interfacial tension vs. the logarithm of the molar concentration of the original and the enzymatically modified phospholipids. pl, original phospholipids; p2e, pl modified by hydroxyoleic acid; p3e, pl modified by hydroxystearic acid; p6e, pl modified by epoxyoleic acid.

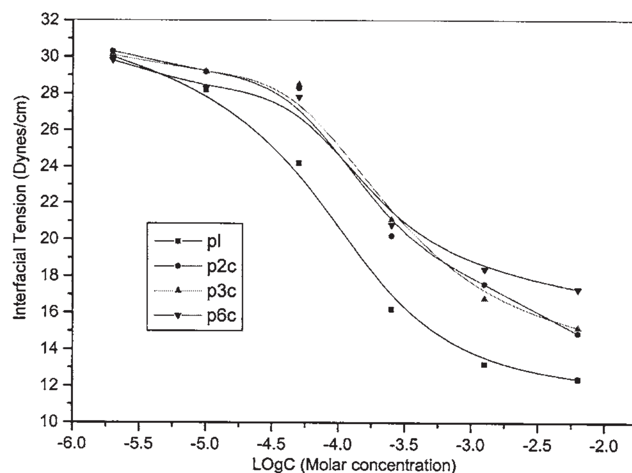

FIG. 5. Plot of interfacial tension vs. the logarithm of molar concentration of original and chemically modified phospholipids. pl, original phospholipid; p2c, pl modified by hydroxyoleic acid; p3c, pl modified by hydroxystearic acid; p6c, pl modified by epoxyoleic acid.

the original soy phospholipids. The presence of two polar groups, such as hydroxy or epoxy, at both positions *sn*-1 and *sn*-2 leads to a kind of branching effect in modified products. As a result, the interfacial tension values of the modified products became different from those of the original soy phospholipids. The chemically modified products showed lower interfacial values compared with the corresponding enzymatic ones. This may be due to the difference in the proportion of the oxygenated FA between the *sn*-1 and *sn*-2 positions. The oxygenated FA in the chemically modified products occupied the *sn*-2 positions in relatively greater proportion than those of the enzymatic products; the latter, as expected, had more of the oxygenated acids at the *sn*-1 position.

Figures 4 and 5 are plots of interfacial tension (γ) vs. the logarithm of molar concentration ($\log C$) of enzymatically and chemically modified soy phospholipids, respectively. The slopes of the plots for some distance below the CMC are linear, indicating the maximum surface excess concentration was reached. CMC values are taken as the molar concentration at the point of the curve g vs. $\log C$ at which interfacial tension changes abruptly. The CMC value of original soy phospholipid is 5.16×10^{-5} M/L. The percent incorporation of polar group (hydroxy and epoxy) is less in chemically modified soy phospholipids than in enzymatically modified soy phospholipids. The CMC values of chemically modified products are closer to the original soy phospholipids. But there is a sharp change in CMC values of enzymatically modified products, presumably due to more incorporation. The CMC values of enzymatically modified products are much lower than chemically modified soy phospholipids. The lower CMC values of the enzymatically modified soy phospholipids revealed that they have stronger aggregation ability than chemically modified products. It appears that the hydrophobic group has the most pronounced effect on the CMC values. In the glucamide series, the sharp decrease in CMC value was seen with increasing alkyl chain length (16). In case of both enzymatic and chemical modification, the effect of the molecular size appears to have a minor effect on the CMC value.

The surface excess concentration (Γ_{max}), in mol/cm^2, and minimum area/molecule (A_{min}), in Å^2, were calculated (15) from Equations 2 and 3,

$$\Gamma_{max} = \frac{1}{2.303\, nRT}(\partial\gamma/\partial\log C)_{max,T} \quad [2]$$

$$A_{min} = 10^{16}/N\,\Gamma_{max} \quad [3]$$

where $n = 1$ (phospholipids is a amphipile molecule and it is a mixture of different phosphatides) and $(\partial\gamma/\partial\log C)_{max}$ is the maximum slope in each case, T is absolute temperature, $R = 8.31 \times 10^7$ erg mol^{-1} K^{-1}, and N is Avogadro's number. The γ_{CMC} values of the original and modified phospholipids are also listed in the Table 2. It is clear from the literature that Γ_{max} increases and A_{min} decreases slightly but steadily with increase in acyl group length from C_{11} to C_{14} in N-methyl glucamides (16). In the present case, the change of Γ_{max} values (also A_{min}) of modified soy phospholipids from those for the original soy phospholipids could be due to the effect of structural difference of the incorporated acids in each case. The presence of hydroxy and epoxy groups in soy phospholipids facilitated the interaction with water to a greater extent than that of the original soy phospholipids. The modified soy phospholipids tended to go to the solution side from the interface and resulted in the lowering of the surface excess concentration value (Γ_{max}) compared with that of the original soy phospholipids. The Γ_{max} values varied depending on the nature of the oxygenated FA incorporated in the soy phospholipids.

The standard free energy change of micellization of surfactant, when the CMC value is less than 1×10^{-2} M, can be calculated by Equation 4,

$$G^{o}_{mic} = RT \ln \text{CMC}/\omega \quad [4]$$

where CMC is expressed in molar units, ω is the number of moles of water per liter of water, T is the absolute temperature at which CMC value is determined, and R = 8.31 J mol^{-1} K^{-1}. The G^{o}_{mic} values of enzymatically modified soy phospholipids were slightly different from the chemically modified soy phospholipids in Table 2. There was a small decrease in the negative value of G^{o}_{mic} as the number of hydrophilic units increased.

The SD of a set of observations is calculated by using the Equation 5 (17)

$$\text{SD}(\sigma) = [1/n \sum (X_i - \underline{X})^2]^{1/2} \quad [5]$$

where n = number of observations, here n = 3, X_i = set of observation, $\underline{X}$ = arithmetic mean of the observation.

Soy phospholipids having hydroxy and epoxy FA could be produced by a transesterification reaction using either a lipase catalyst or a chemical catalyst. The modified phospholipids showed distinct changes in surface properties such as effectiveness of interfacial tension reduction (γ_{CMC}), CMC, Γ_{max}, A_{min}, and free energy change of micellization (ΔG^{o}_{mic}). The modified soy phospholipids may find new applications as surface-active agents.

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