Preparation of Polyunsaturated Phospholipids by Lipase-Catalyzed Transesterification

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Transesterifications were investigated to determine a means for preparing polyunsaturated phospholipids simply from soy phospholipid, sardine oil, and two kinds of microbial lipases originating from *Candida cylindracea* and *Rhizopus delemar*.

The optimum reaction conditions for *Candida cylindracea* lipase were: 4 g of sardine oil, 10 mL of water, 0.7 g of lipase, 10 mL of hexane, 48 hr of reaction time at 37°C for 3 g of soy phospholipid, for which the transesterification ratio reached approximately 45%. Recovery of phospholipid was low, because hydrolysis also occurred under these reaction conditions. However, hydrolysis could be suppressed by using glycerine instead of water, and the recovery of phospholipid increased to 47%, although the transesterification ratio was reduced to 32%.

***Rhizopus delemar* lipase has 1,3-specificity for triglycerides, and the transesterification ratio was approximately 37% in the 1-position of phospholipid. The resulting phospholipid was rich in polyunsaturated fatty acids and linoleic acid, while the total percentage of polyunsaturated fatty acids incorporated was 18.4%. Therefore, polyunsaturated phospholipids can be prepared easily by transesterification of soy phospholipid with fish oil by means of commercial lipases.**

KEY WORDS: Fish oil, lipase, phospholipids, polyunsaturated fatty acids, transesterification.

Certain polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are biologically active substances that inhibit platelet aggregation and prevent cerebral thrombosis and arterial sclerosis (1–4), have attracted attention. These acids have been used hitherto in health foods and drugs as their ethyl esters, but it has been clarified recently that the acids are absorbed much better as glycerides (5,6).

On the other hand, the physiological activity of phospholipids (PL), such as some fish PL that are rich in PUFA (7), has also attracted much interest. K. Yazawa *et al.* (8,9) reported that polyunsaturated phospholipids (PUFA-PL) produced by enterobacteria in certain fishes reduce levels of neutral fat or cholesterol. In the present work, we tried to develop simple and rapid preparation of PUFA-PL by lipase-catalyzed transesterification between soy PL and fish oil, Japan's only exported oil resource.

EXPERIMENTAL PROCEDURES

Materials. Commercially purified soy PL (The Nissin Oil Mills Ltd., Yokohama, Japan) and sardine oil (Nippon Oil & Fats Co. Ltd., Tokyo, Japan) were used as raw materials in transesterification; their fatty acid compositions are shown in Table 1.

TABLE 1

Fatty Acid Composition of Soy PL and Sardine Oil (%)

Fatty acid	Soy PL	Sardine oil
C14:0	—	6.0
C16:0	21.9	13.8
C16:1	—	4.0
C18:0	2.1	6.3
C18:1	9.3	24.0
C18:2	59.0	1.0
C18:3	7.7	—
C18:4	—	1.9
C20:1	—	5.6
C20:4	—	0.8
C20:5	—	11.2
C22:1	—	6.5
C22:4	—	1.2
C22:5	—	2.6
C22:6	—	9.8
Others	—	5.3

Lipase OF with 360,000 units/g of activity, obtained from *Candida cylindracea* (Meito Sangyo Co. Ltd., Tokyo, Japan), which is considered a random lipase, and lipase originated from *Rhizopus delemar* with 6,000 units/mg (Seikagaku Kogyo Co. Ltd., Tokyo, Japan), which has 1,3-positional specificity for triglyceride (TG), were used as enzymes in bio-catalyzed transesterification.

Transesterification. Transesterification reactions with *Candida cylindracea* lipase were carried out over fixed time periods at 37°C and at 350 rpm in a rotary shaker model SCS-T (Iwashiya Co. Ltd., Tokyo, Japan) in 100 mL Erlenmeyer flasks containing 3 g of soy PL, varying amounts of sardine oil dissolved in 10 mL of hexane, 0.7 g of lipase, and fixed amounts of either deionized water or glycerine. The acyl group molar ratio of sardine oil to PL was 1.5 when 3 g of PL and 4 g of sardine oil were used. Transesterified PL was recovered from the reaction mixture as follows: first the hexane was removed, the residue was extracted with 50 mL of Folch solvent (methanol/chloroform = 2/1), the solvent was evaporated, and then the residue was recrystallized from acetone to remove TG (with the acetone) and leave PL behind.

Transesterification reactions with *Rhizopus delemar* lipase were carried out with 1 g of soy PL, fixed amounts of sardine oil, hexane, 0.05M acetic acid buffer solution (pH 5.6), and 0.1M calcium chloride at 350 rpm and at 40°C. The transesterified PL was recovered by the same procedure as described above.

Analyses of transesterified PL. Thin-layer chromatography-flame ionization detector (TLC-FID) analysis confirmed that transesterified PL contained no TG. Analytical conditions for the TLC-FID were as follows: Iatroscan model TH-10 with silica gel rod S III (Iatron Laboratories, Inc., Tokyo, Japan) as equipment, and benzene/chloroform/acetic acid = 35/15/1 as the solvent mixture for developing.

Gas-liquid chromatography (GLC) analysis was done

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also to calculate transesterification ratios of recovered PL based on the fatty acid composition between original and recovered PL. The transesterification ratio was defined as the percentage of fatty acids that were absent as constituents of the original PL and were incorporated into the recovered PL. Methyl esters of fatty acids constituting the PL for GLC analysis were prepared according to Jham *et al.* (10), that is, 50 mg of the PL were put into a 15-mL test tube with screw cap and dissolved in 1 mL of benzene, 1 mL of 0.5N potassium hydroxide-methanol solution was added and the mixture was heated for 5 min at 100°C in an aluminum block. Then, 0.4 mL of 9.6N hydrochloric acid-methanol solution was added to the test tube, which was heated again at 100°C for 15 min. The test tube was then cooled to room temperature, 2 mL each of deionized water and hexane were added, and the esters were washed several times with deionized water, dried with anhydrous sodium sulfate, and concentrated by evaporation. A model GC-8A (Shimadzu Corp., Kyoto, Japan) gas chromatograph was employed that was equipped with two glass columns packed with 15% DEGS on celite (3 mm i.d. × 2 m length). Helium carrier was used at 40 mL/min.

RESULTS AND DISCUSSION

Optimum amounts for the lipase and hexane in the transesterification with *Candida Cylindracea* lipase were found to be 0.7 g and 10 mL, respectively when compared with 0.3, 0.5 and 0.9 g for the lipase, and with 5, 15, 20 mL for the hexane, when 3 g of soy PL was used.

The transesterification ratio of PL increased gradually up to 44% over 48 hr, and after that time it reached a plateau.

The influence on the reaction of added water is shown in Figure 1. This showed that 10 mL of water was the optimum amount for 3 g of soy PL. This also suggested, however, that hydrolysis had been occurring along with transesterification, because the recovery ratio of PL decreased gradually as the water was increased.

Relationships between the ratio of acyl groups of sardine oil/PL, the ratios of transesterification and of PL recovery were investigated; the results are shown in Figure 2. We found that the transesterification ratio gradually increased with increases in sardine oil added, while the recovery of PL was nearly constant at 20%. When the reaction was performed with a ratio of 3/1 in acyl groups of sardine oil/PL, the transesterification ratio reached 75%. It was assumed that all acyl groups hydrolyzed with *Candida cylindracea* lipase were introduced randomly into PL. Therefore, a transesterification ratio of more than 70% was estimated from calculations based on the theoretical value of 75% in the present reaction. We believe that the transesterification would increase to a higher ratio by increasing the percentage of acyl groups derived from sardine oil per PL. As mentioned above, however, hydrolysis of PL has undoubtedly occurred concurrently with transesterification, judging from the low (approximately 20%) recoveries of PL. Accordingly, the transesterification was performed with glycerine instead of water to suppress hydrolysis. The highest transesterification ratio obtained was 32%, even with 10 mL of glycerine, although the recovery of PL increased from approximately 20% with water to more than 40% with glycerine, as shown in Figure 3.

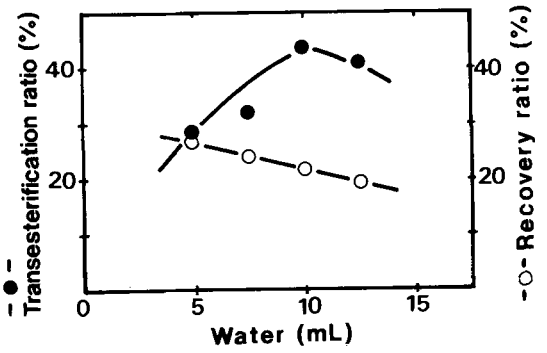

FIG. 1. Effects of water content on transesterification of PL with *Candida cylindracea* lipase. Reaction conditions: 3 g PL, 4 g sardine oil, 0.7 g lipase, 10 mL hexane, reaction time 48 hr and reaction temperature 37°C.

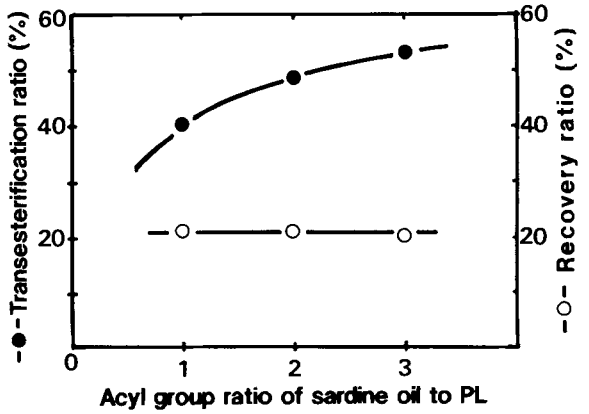

FIG. 2. Changes of transesterification ratio of PL as function of acyl group ratio of sardine oil to PL. Reaction conditions: 3 g PL, 10 mL water, 0.7 g lipase, 10 mL hexane, reaction time 48 hr and reaction temperature 37°C.

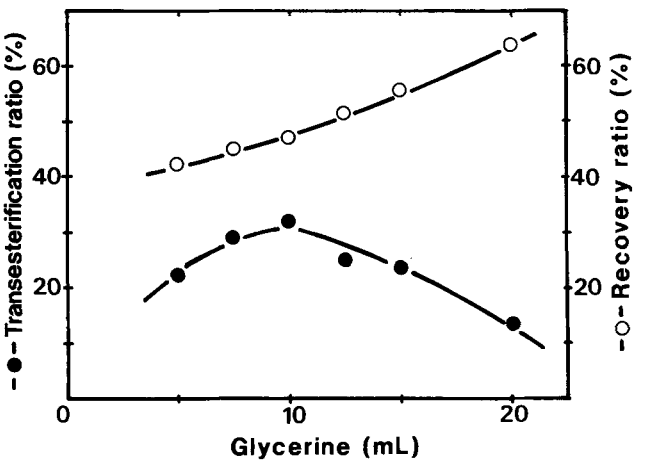

FIG. 3. Effects of glycerine on transesterification of PL with *Candida cylindracea* lipase. Reaction conditions: 3 g PL, 4 g sardine oil, 0.7 g lipase, 10 mL hexane, reaction time 48 hr and reaction temperature 37°C.

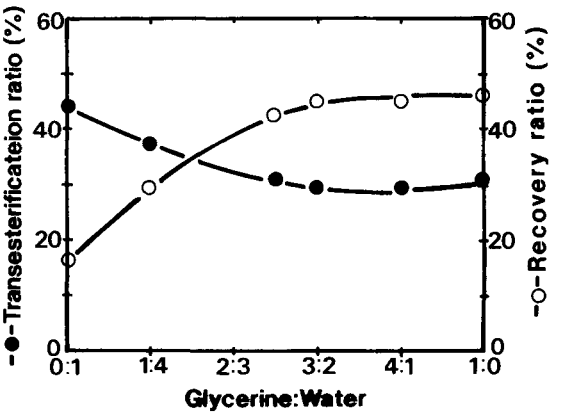

FIG. 4. Effects of mixed ratio of glycerine and water on transesterification of PL with *Candida cylindracea* lipase. Reaction conditions: 3 g PL, 4 g sardine oil, 0.7 g lipase, 10 mL hexane, reaction time 48 hr and reaction temperature 37°C.

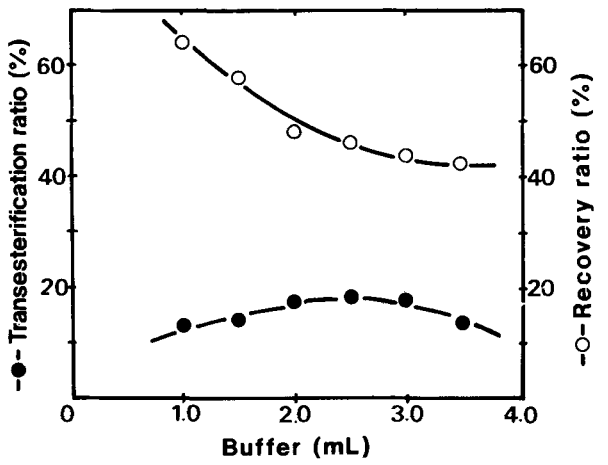

FIG. 5. Effects of water content in buffer on transesterification of PL with *Rhizopus delemar* lipase. Reaction conditions: 1 g PL, 1.3 g sardine oil, 0.03 g lipase, 3.3 mL hexane, reaction time 24 hr and reaction temperature 40°C.

Other attempts were also made to modify the reaction with water-glycerine mixtures, and the results are shown in Figure 4. It is obvious from Figure 4 that the recovery of PL increased with a decrease in water content in the solution from 0:1 through 3:2 and then remained constant afterward. On the other hand, the transesterification ratio decreased with decreasing levels of water before reaching a constant state; the reverse was true for the changes in the recovery ratio. Therefore, we believe that a higher transesterification ratio requires a higher water content in the reaction system, but that a lower water content in the water-glycerine solution also results in lower transesterified PL with higher recovery.

The optimum conditions for transesterification with *Rhizopus delemar* lipase were investigated with 1 g of soy PL, 1.3 g of sardine oil, 3.3 mL of hexane, and 3.3 mL of 0.05M acetate buffer solution (pH 5.6). The transesterification ratio increased rapidly for 10 hr, then increased gradually, and remained constant after 24 hr.

The optimum enzyme amount was 0.03 g because the transesterification ratio increased from 0.01 through 0.03 g, but remained constant beyond that. Figure 5 indicates the optimum water concentration for 0.03 g of enzyme and a reaction time of 24 hr. In this case, 18.4% transesterification was obtained with 2.5 mL of buffer solution. It can be estimated that the transesterification ratio of the 1-position for PL reaches approximately 37% if transesterification occurs only in the 1-position of the PL, because *Rhizopus delemar* lipase has 1,3-positional specificity for TG. The recovery of PL decreased as the water level increased because hydrolysis occurred concurrently as in the experiments with *Candida cylindracea* lipase.

TABLE 2

Fatty Acid Composition of Transesterified PL with Two Lipases Originated from *Candida cylindracea* and *Rhizopus delemar*

Lipase	*Candida cylindracea*		*Rhizopus delemar*
Reaction condition	Water	Glycerine	Buffer (pH 5.6)
Transesterification ratio (%)	44.4	31.8	18.4
Recovery ratio of PL (%)	17.0	46.8	46.9
Fatty acid composition (%)			
C18:2	17.9	24.6	49.6
C20:4	2.5	1.8	1.3
C20:5	14.5	11.2	3.6
C22:6	3.1	—	2.3
Others	62.0	62.4	43.2
Average degree of unsaturation	3.6	3.0	4.0

The fatty acid compositions of the transesterified PL are given in Table 2, with special attention given to linoleic acid (LA), arachidonic acid (AA), EPA and DHA. The LA content was approximately 60% in the original soy PL and decreased to 17.9% or 24.6% when reactions were conducted with *Candida cylindracea* lipase and water or glycerine, and 14.5% or 11.2% of EPA was incorporated into PL, respectively. Transesterified PL obtained with *Rhizopus delemar* lipase included 50% LA, 3.6% EPA and 2.3% DHA as PUFA, although the transesterification ratio was only 18.4%. The average degree of unsaturation of PL obtained with *Rhizopus delemar* lipase was higher than that with *Candida cylindracea* lipase. EPA and DHA contents of transesterified PL were not high either with *Candida cylindracea* lipase or with *Rhizopus delemar* lipase in these experiments, since both reactions were performed with sardine oil, which contained only 2.6% EPA and 9.8% DHA. It is, however, believed that a higher unsaturated PL could be obtained with EPA- and DHA-concentrated fish oil (11,12) as PUFA substrates. In addition, PUFA-PL with different fatty acid compositions could be prepared by using lipases from different origins.

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