Modification of Soybean Phospholipids by Enzymatic Transacylation

Setsuko HARA^{*1}, Hiroyuki HASUO^{*1}, Makoto NAKASATO^{*2},
Yuzo HIGAKI^{*2} and Yoichiro TOTANI^{*1}

^{*1} Faculty of Engineering, Seikei University

(3-1 Kichijoji-kitamachi 3, Musashino-shi, Tokyo 180-8633, JAPAN)

^{*2} The Nisshin Oil Mills, Ltd.

(Shinmori-machi 1, Isogo-ku, Yokohama, 235-8558, JAPAN)

Edited by K. Kikugawa, Tokyo Univ. Phar. Life Sci., and accepted January 16, 2002 (received for review October 19, 2001)

Abstract: Modified glycerophospholipids with expected novel physiological functions were prepared by introducing different acyl groups into soybean phospholipids. The method was based on the enzymatic transesterification of phospholipids with monoacylglycerol (MG) or diacylglycerol (DG). The transacylation ratios reached 46-59% and nearly quantitative recovery of phospholipids was obtained in the case of transacylation of phospholipids with MGs having C12 to C18 saturated and monounsaturated acyl groups. With C18:2-MG or C18:3-MG, however, transacylation ratios were less than ca. 20%. MGs with C4 to C8 saturated acyl groups also gave very low transacylation ratios, but the ratios were moderately improved by using DGs with C6 and C8 acyl groups. It is considered from a series of the results that higher transacylated phospholipids might be obtained by repeating transacylations owing to be able to recover the phospholipids quantitatively after the reaction because MG with longer acyl groups than C10 or DG with C8 acyl groups acted as acyl donors and replacement for essential water, to suppress the simultaneous hydrolysis, in an enzymatic reaction.

J. Oleo Sci., **51**, 417-429 (2002).

Key words: phospholipid, lipase, transacylation, monoacylglycerol, diacylglycerol, fatty acid

1 Introduction

Phospholipids are on the market as health promoting supplemental food additives. Many physiological activities are claimed and phospholipids are used as typical natural antioxidants for foods, cosmetics, medicines, and others (1). The development of drug delivery systems and artificial blood are also carried out by utilization of the binary hydrophilic – lipophilic characteristics of phospholipids (2). Up to the present, many research studies for the reforming and fractionation of phospholipids were performed on phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA). Nevertheless,

there are still many unknown effects of the acyl group of phospholipids on property and function, since commercially available phospholipids are soybean phospholipids consisting mainly of linoleic acid. Egg yolk phospholipids consist mainly of palmitic acid, and other commercially prepared phospholipids also contain saturated fatty acids.

In the present work, modified glycerophospholipids which are expected to have some novel physiological functions were prepared as shown in **Fig. 1**.

Different acyl groups were introduced into soybean phospholipids as described in our previous report (3). Transesterification of phospholipids, in which monoacylglycerol (MG) acted as an improved acyl donor to introduce an acyl group into soybean phospholipid and replaced the essential water to maintain enzyme activity, was proposed.

Corresponding author: Setsuko HARA

E-mail: shara@ch.seikei.ac.jp

Fig. 1 Transacylation of Phosphatidylcholine with Lipase OF or Lipase SM-10.

2 Experimental

2.1 Materials

A soybean phospholipid which is about 70% phosphatidylcholine (PC-70) was obtained from Nisshin Oil Mills Ltd, Tokyo. Egg yolk phospholipid was purchased from Wako Pure Chemical Industries, Ltd. Osaka. The lipid compositions and purities of soybean and egg yolk phospholipids used in the present work are shown in **Table 1**, and fatty acid compositions of the two phospholipids and their PCs are given in **Table 2**.

Since those two phospholipids consist mainly of PC in their phospholipid constituents as shown in **Table 1**, the transacylation ratio was calculated for only PC, although it was considered that the reaction could also proceed in other component phospholipids than PC.

Various MGs and diacylglycerols (DGs) such as monolinolenin (C 18:3-MG), monolinolein (C 18:2-MG), monoolein (C 18:1-MG), monostearin (C 18:0-MG), monopalmitin (C 16:0-MG), monomyristin (C 14:0-MG), monolaurin (C 12:0-MG), monocaprin (C 10:0-MG), monocaplin (C 8:0-MG), monocaproin (C 6:0-MG), monobutylin (C 4:0-MG), dicaplin (C 8:0-DG), dicaproin (C 6:0-DG), and dibutylin (C 4:0-DG) were used in the present experiment and those acylglyc-

erols except for C 18:2-MG were prepared by esterification of glycerol with corresponding fatty acids and concentrated sulfuric acid as a catalyst (4) where needed. C 18:2-MG was obtained commercially. Fatty acid compositions and purities of various MGs and DGs are shown in **Table 3**.

Lipases used were Lipase OF originating in *Candida rugosa* (360 U/mg, Meito Sangyo Co. Ltd., Aichi) and an immobilized Lipase SM-10 from *Rhizopus oryzae* (120 U/mg) donated by Dr. Y. Shimada of Osaka Municipal Technical Research Institute (Osaka). Other reagents, solvents and chemicals were of reagent grades and were used without further purification.

2.2 Transacylation with Lipase OF or Lipase SM-10

The transacylation reaction with Lipase OF which has no regio specificity was carried out as follows: 0.6 g of soybean phospholipid, six molar of MG for one molar of soybean phospholipid (3 times excess acyl groups supplied from MG against two acyl groups of phospholipid), 140 mg of Lipase OF and 3 mL of hexane, were stirred in a 30 mL Erlenmeyer flask for 48 h at 37°C. The reaction with Lipase SM-10, 1,3-regio specific lipase, to prepare a modified phospholipid having lauroyl group in *sn*-1 position as a main acyl group in a molecule was performed under similar reaction conditions as in the case of Lipase OF, except for the lipase activity level.

2.3 Isolation and Characterization of Lipids

Lipids and fatty acid compositions of starting materials and reaction products were analyzed with TLC-FID (Iatroscan model MK-5, Dia-Iatron Co. Ltd., Tokyo)

Table 1 Lipid Composition of Soybean and Egg Yolk Phospholipids(PL)(%).

	Soybean PL (PC-70)	Egg Yolk PL
Phosphatidylcholine	65.9	65.4
Phosphatidylethanolamine	7.9	12.2
Phosphatidylinositol	5.0	1.1
Others	21.2	21.3
PL purity	79.8	79.3

Table 2 Fatty Acid Composition of Soybean and Egg Yolk Phospholipids (PL) and their Phosphatidylcholines (PC) (%).

Fatty Acids	Soybean PL (PC-70)	Soybean PC	Egg Yolk PL	Egg Yolk PC
C14:0		2.8	0.6	
C16:0	15.4	21.0	31.2	35.5
C18:0	8.0	3.8	11.5	7.0
C18:1	8.8	16.9	40.4	24.3
C18:2	61.5	52.4	11.9	7.6
C18:3	6.3	3.1	0.5	7.6
Others			3.9	18.0

and capillary GLC (Shimadzu model GC-18, Kyoto), respectively. Developing solvents in TLC-FID were benzene / chloroform / acetic acid = 50/20/0.7(v/v/v) to determine lipid composition and chloroform / methanol / acetic acid = 65/25/10(v/v/v) for phospholipid composition determinations. Prior to GLC, lipid fatty acids were methyl-esterified in accordance with the method reported by Jham *et al.* (5). After the transesterification, total phospholipids including transacylated and untransacylated phospholipids were separated from simple lipids such as free fatty acids, MG or DG by the acetone fractionation, and then PC was recovered from the phospholipids mixture by preparative TLC with a 2 mm thick plate of silica gel 60 (10×10 cm; Merck, Darmstadt, Germany) and chloroform / methanol / acetic acid / water = 100/80/15/6 (v/v/v/v) as a mobile phase. Transacylation ratios were calculated with the equation shown below on the basis of the difference between the fatty acid composition of PC before and after the reaction.

$$\text{Transacylation ratio (\%)} = (A - B) \times 100 / C$$

where A: % of the acyl group content corresponding to acyl donor in the recovered PC

B: % of the acyl group content corresponding to acyl donor in the original PC .

C: % of acyl group purity of the acyl donor.

3 Results and Discussion

3.1 Transacylation of Phospholipids with Different Chain Lengths of MGs and Lipase OF

The transacylation ratios of soybean PC and C 4 to C 18 saturated MGs were 2.3 for C 4, 1.8 for C 6, 7.6 for C 8, 28.1 for C 10, 54.8 for C 12, 55.7 for C 14, 48.4 for C 16, and 59.8% for C 18 as shown in **Table 4**. From these results, it was confirmed that long chain saturated acyl groups such as C 14 to C 18 were introduced successfully into the PC. Although it is not clear why the C 4 to C 8 acyl groups were incorporated at such a low

Table 3 Fatty Acid Composition and Purity of Monoacyl Glycerols (MG) and Diacyl Glycerols (DG) (%).

	C4:0-MG	C6:0-MG	C8:0-MG	C10:0-MG	C12:0-MG	C14:0-MG	C16:0-MG	C18:0-MG	C18:1-MG	C18:2-MG	C18:3-MG	C6:0-DG	C8:0-DG	C10:0-DG	C12:0-DG
C4:0	100.0														
C6:0		100.0										100.0			
C8:0			100.0										100.0		
C10:0				99.8										100.0	
C12:0					99.8										98.9
C14:0				0.2		98.5			0.3						1.1
C16:0					0.2	1.2	99.1	2.5	9.0	0.7	7.3				
C18:0						0.3	0.9	96.0	2.1	1.0	0.2				
C18:1								1.3	88.6	2.1	0.8				
C18:2								0.2		96.2					
C18:3											91.7				
Purity	98.2	100.0	98.5	98.8	97.8	99.0	98.5	97.9	97.6	99.1	61.3	90.5	89.3	99.1	92.3

level into the PC, it was considered that hydrophilic-lipophilic balance (HLB) affects the transacylation. The reaction of PC-70 with highly purified C 6 to C 12 saturated DGs was then carried out by using Lipase OF. As shown in **Table 4**, C 6 and C 8 DGs which are more lipophilic than the corresponding MG, were 4 times more effective in obtaining higher transacylation ratios, although C 10-DG and C 12-DG showed less effect on the ratios in comparison with the corresponding MGs. Therefore, DGs with C 6 or C 8 groups are considered to be more effective acyl donors than those of C 6-MG or C 8-MG for the transacylation of phospholipids.

3.2 Transacylation of Phospholipids with C 18-MGs and Lipase OF

Total phospholipids including transacylated and untransacylated phospholipids were recovered almost quantitatively after the reaction.

Linoleic acid, the main component of soybean PC, decreased and the donor acyl group were introduced into the PC by the transacylation of PC with different C 18-MGs and Lipase OF except for the reaction between soybean PC and C 18:2-MG as shown in **Table 4**. Acyl groups between PC and C 18-MGs of monostearin, monoolein, monolinolein, or monolinolenin could be exchanged with 59.8, 54.7, 24.9 or 18.3% of transesterification ratio, respectively. C 18:1-MG was proved to act as an excellent acyl donor like C 18:0-MG. It is considered that the low transacylation ratios of C 18:2-PC and C 18:3-PC were due to the autoxidation of C 18:2-MG and C 18:3-MG during the present

experiment because the lipase activity is deteriorated by lipid peroxides (6). Furthermore, it is probable to deactivate enzymes by the oxidation of enzyme proteins since there were many reports on the denaturalization of proteins or amino acids by lipid peroxides (7-10).

The transacylation of egg yolk phospholipids with C 18:2-MG under the same condition was carried out to check why the linoleoyl group could not be introduced into the PC. As a result, linoleic acid content in the egg yolk PC was increased from 7.6% to ca. 28% by the transacylation, and the transacylation ratio was calculated as 21.2%. There is, however, little differences between the results obtained with soybean and egg yolk PCs. Therefore, further experiments are necessary to clarify why linoleic acid could not be introduced into soybean or egg yolk PCs.

3.3 Transacylation of Phospholipids with Monolaurin and Lipase SM-10

Since it was expected to prepare another modified PC by incorporating a functional fatty acid into the *sn*-1 position of PC with 1,3-regio specific lipase, we tried the transacylation of PC-70 with C 12:0-MG and Lipase SM-10 which is a 1,3-regio specific immobilized *Rhizopus oryzae* lipase, as a model experiment.

As results of the examination for the optimum catalytic amount of Lipase SM-10 in the transacylation between PC-70 and C 12:0-MG by using 460 to 20,000 units of Lipase SM-10, the maximum transacylation ratio of 37.5% was obtained at 7,840 units of Lipase SM-10 for 48 h at 37°C. The theoretical maximum

Table 4 Fatty Acid Composition and Conversion Ratios of Transacylated Soybean PC with Monoacyl Glycerols (MG) or Diacyl Glycerols (DG) with Lipase OF (%).

	C4:0-MG	C6:0-MG	C8:0-MG	C10:0-MG	C12:0-MG	C14:0-MG	C16:0-MG	C18:0-MG	C18:1-MG	C18:2-MG	C18:3-MG	C6:0-DG	C8:0-DG	C10:0-DG	C12:0-DG
C4:0	2.3														
C6:0		1.8										3.2			
C8:0			7.6										29.6		
C10:0				27.9										18.1	
C12:0			1.0	1.1	54.7										22.1
C14:0	1.1	1.6			0.5	57.7	1.0	1.1			1.3	1.1	0.3		0.3
C16:0	7.8	16.9	2.3	14.6	2.8	8.2	69.0	8.8	6.5	2.5	19.2	19.8	13.2	16.6	14.8
C18:0	4.0	0.8	27.1	3.1	1.4	1.2	1.7	61.2	0.5	15.7	3.3	4.1	5.6	8.3	2.9
C18:1	17.1	16.0	0.6	8.3	3.2	5.0	7.4	7.7	65.4	0.5	15.0	15.5	9.9	5.5	11.3
C18:2	51.2	42.3	50.6	39.9	29.5	24.9	20.1	20.0	27.5	76.4	41.3	54.7	39.3	49.6	48.2
C18:3	10.4	20.6	10.8	5.1	7.8	3.0	0.8	1.2	0.1	4.9	19.9	1.5	2.1	1.9	0.3
Ratio	2.3	1.8	7.6	28.1	54.8	55.7	48.4	59.8	54.7	24.9	18.3	3.2	29.6	18.1	22.3

transacylation ratio of PC is expected to reach 50%, as Lipase SM-10 has 1,3-regio specificity. The ratio of 37.5% obtained in the present experiment was equal to 75% of the theoretical value. Therefore, Lipase SM-10 is found to be an effective lipase for preparation of transacylated PC in the *sn*-1 position. On the other hand, Lipase OF is considered to be available to exchange the all acyl groups in PC.

4 Conclusion

It was reported by H. Brockerhoff *et al.* in 1976 that lipase could be used for transacylation (11), although the enzymatic reforming of phospholipids was usually performed with specific phospholipases. Polyunsaturated fatty acids were introduced into phospholipids with chemically modified lipase by Yoshimoto *et al.* (12), and the authors have also reported on the transesterification of soybean phospholipid with fish oil rich in docosahexaenoic acid (13). Hosokawa and Takahashi *et al.* have achieved the introduction of 31% eicosapentaenoic acid into PC (14), but a practical process to prepare a functional phospholipid was necessary, as the recovery of reformed phospholipids in those studies was at very low levels.

In the present work, the transacylation ratios of soybean PC reached 48–60% and nearly quantitative recoveries of the total phospholipids were obtained in the case of transacylation of PC-70 with MGs having C 12 to C 18 saturated acyl and octadecenoyl groups. With C 18:2-MG or C 18:3-MG, however, those transacylation ratios were about 20%. MGs with C 6 and C 8 saturated acyl groups also gave very low transacylation ratios, but the ratios were moderately improved by using DGs with those acyl groups. In any case, it was difficult to incorporate short chain acyl groups such as C 4 or C 6 into the soybean PC.

Several MGs acylated with C 12 to C 18 saturated acid and DGs with C 6 and C 8 saturated acid efficiently acted as acyl donors in the present transacylation. In addition, because a reaction system presented in this study did not require the addition of water essential for the activation of enzymes, the simultaneous hydrolysis was suppressed and phospholipids were able to recover almost quantitatively. We have reported in the previous paper (15) that the repeating transacylation is effective to obtain highly transacylated phospholipids when phospholipids were recovered quantitatively after the

transacylation of soybean phospholipids with polyunsaturated fatty acids such as EPA and DHA, even if the transacylation ratio was nothing but ca. 20%.

It is considered from a series of the present results that higher transacylated phospholipids might be obtained by repeating transacylations owing to be able to recover the phospholipids almost quantitatively after the reaction because MG with longer acyl groups than C 10 or DG with C 8 acyl groups acted as better acyl donors and replacement for essential water, to suppress the simultaneous hydrolysis, in an enzymatic reaction.

Acknowledgments

The authors express their hearty thanks to Dr. Y. Shimada of Osaka Municipal Technical Research Institute who donated lipase SM-10. The authors are grateful to Dr. Lourens Du Plessis of South African National Food Research Institute for his useful discussion and comments.

References

1. Schneider, M. (2001) *Eur. J. Lipid Sci. Technol.*, **103**, 98–101.
2. Scherphof, G., Damen, J. & Hoekstra, P. (1981) *Liposomes: From Physical Structure to Therapeutic Applications* (C.G. Kight ed.) Elsevier, p.299.
3. Nakasato, M., Hara, S. & Totani, Y. (1997) *J. Jpn Oil Chem. Soc. (J. Oleo Sci.)*, **46**, 791–795.
4. Kirby, A. (1972) *Ester Formation and Hydrolysis and Related Reactions* (C.H. Bamford ed.) Elsevier, p.126.
5. Jham, G.N., Teles, F.F.F. & Campos, L.G. (1982) *J. Am. Oil Chem. Soc.*, **59**, 132–133.
6. unpublished data.
7. Little, C. & O'Brien, P.J. (1968) *Biochem. J.*, **106**, 419–423.
8. Gardner, H.W. (1979) *J. Agr. Food Chem.*, **27**, 220–229.
9. Matsushita, S. (1975) *J. Agr. Food Chem.*, **23**, 150–154.
10. Yoshioka, M. & Kaneda, T. (1974) *J. Jpn Oil Chem. Soc. (J. Oleo Sci.)*, **23**, 321–326.
11. Brockerhoff, H., Schmidt, P.C., Fong, W. & Tirri, I. (1976) *Lipids*, **11**, 421–425.
12. Yoshimoto, T., Nakata, M., Yamaguchi, S., Funada, T., Saito, Y. & Inada, Y. (1986) *Biotechnol. Lett.*, **8**, 771–775.
13. Totani, Y. & Hara, S. (1991) *J. Am. Oil Chem. Soc.*, **68**, 848–851.
14. Takahashi, K. (1999) *J. Lipid Nutr.*, **8**, 124.
15. Totani, Y. (1999) *J. Jpn Oil Chem. Soc. (J. Oleo Sci.)*, **48**, 1233–1245.