

Optimization of the Reaction Conditions in the Lipase-Catalyzed Synthesis of Structured Triglycerides

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ABSTRACT: Structured triglycerides of the ABA-type, containing one type of fatty acid (A) in the *sn*-1 and *sn*-3 positions and a second type of fatty acid (B) in the *sn*-2 position of the glycerol, were synthesized using lipases. The highest yields and purities were achieved in a two-step process, where a triglyceride of the B-type was subjected to an alcoholysis reaction in an organic solvent catalyzed by *sn*-1,3-regiospecific lipases yielding the corresponding 2-monoglyceride (2-MG). Using this strategy, e.g., 2-monopalmitin (2-MP) was obtained in up to 88% yield at >95% purity by crystallization. Esterification of 2-MP with oleic acid resulted in the formation of 1,3-oleyl-2-palmitoyl-glycerol in up to 72% yield containing 94% palmitic acid in the *sn*-2 position. The best lipases were from *Rhizomucor miehei*, *Rhizopus delemar*, and *Rhizopus javanicus*. Water activity, solvent, and carrier for lipase immobilization strongly influenced the yield and purity of the products in both steps. Furthermore, 2-MG from fish oil were produced by alcoholysis in up to 84% yield at >95% purity.

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The nutritional value of triglycerides and their physicochemical properties are determined not only by the fatty acid composition, but also by the positional distribution of the acyl groups bonded to the glycerol. Special triglycerides of the ABA-type containing medium-chain fatty acids (e.g., C₈) in *sn*-1,3 positions and a long-chain unsaturated fatty acid (e.g., C₁₆–C₂₂) in the *sn*-2 position are an effective energy source for patients with malabsorption, e.g., pancreatic insufficiency (1). Synthesis, biochemistry, and use of structured triglycerides were recently documented (2). An important structured triglyceride in infant nutrition is 1,3-oleyl-2-palmitoyl-glycerol (OPO). Human milk contains palmitic acid predominantly in the *sn*-2 position of triglycerides, but most infant

formulas contain palmitic acid predominantly in *sn*-1,3 positions, and their release during digestion may cause the formation of poorly absorbed calcium soaps. In contrast, fats containing palmitic acid exclusively at the *sn*-2 position are absorbed more efficiently (3).

Polyunsaturated fatty acids (PUFA) like eicosapentaenoic acid (EPA, C_{20:5}) and docosahexaenoic acid (DHA, C_{22:6}) have been reported to have several advantages compared to conventional fatty acids, such as reduction of blood pressure and plasma triglyceride levels, and control of overactive immune functions (4). DHA is recognized as being important for brain and eye development in infants (5). It might be advantageous to provide diets containing only one of these PUFA, because in some studies negative effects on growth were reported when DHA was fed to infants in combination with high EPA levels (5).

The common route reported in literature for the synthesis of structured triglycerides is based on a simple lipase-catalyzed transesterification between two different triglycerides or between a triglyceride and fatty acids (6–8). Unfortunately, yields of ABA-type triglycerides are low and a variety of by-products is formed. These are difficult to separate from the desired product. Recently, we developed an efficient alternative for the synthesis of structured triglycerides based on a two-step process (9,10). First, triglycerides of the B-type were subjected to alcoholysis catalyzed by *sn*-1,3-regiospecific lipases yielding the corresponding 2-monoglycerides (2-MG). The 2-MG were isolated by crystallization and esterified with the A-type fatty acid using the same lipases to form the desired structured triglycerides. This concept was successfully used in the synthesis of MLM-type triglycerides starting from triolein, trilinolein, peanut oil, or cottonseed oil followed by esterification of the corresponding 2-MG with caprylic acid. The alcoholysis was performed in methyl-*t*-butyl ether and the esterification in *n*-hexane. In the present paper, we extended this method to the synthesis of OPO and also prepared 2-MG from fish oil containing high concentrations of PUFA by alcoholysis. Special emphasis was given to the influence of water activity, solvent, and choice of carrier material for the immobilization of the lipases on product yield and purity during both reactions.

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MATERIALS AND METHODS

Lipase. Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) were from *Rhizopus delemar* (RDL), *Rhizopus javanicus* (RJL), *Rhizopus niveus* (RNL), and *Candida rugosa* (all from Amano Pharmaceutical Co. Ltd., Nagoya, Japan). One commercial lipase from RML (Lipozyme IM), immobilized on anion exchange resin, was from Novo (Bagsvaerd, Denmark). All chemicals and solvents used were reagent grade and purchased from common commercial suppliers, with the exception of EP 100, a polypropylene carrier (particle size 200–400 μm) (Akzo, Obernburg, Germany) and Celite 545 (Fluka, Buchs, Switzerland). Fish oil was supplied by Unilever (Bedford, United Kingdom).

General methods. Determination of hydrolytic activity of lipases, immobilization of lipases on Celite or EP 100, esterification of 2-MG, and purification of structured triglycerides were performed as described previously (9).

Alcoholysis of triglycerides. Alcoholysis of fish oil and tripalmitin was essentially performed as described previously (9) with slight modifications. Tripalmitin (0.35 mmol) was dissolved in the solvent given in Table 1 and equilibrated to a water activity of 0.11 over saturated salt solutions in a closed vessel. Then the reaction was started by addition of 10% (based on the weight of triglyceride) of immobilized RML, RDL, RNL, or RJL, pre-equilibrated at water activity 0.11 over saturated LiCl. Dry ethanol (dried over molecular sieve 3Å, which was activated by heating overnight to 250°C) was added to a final concentration of 3.5 mmol. The reaction was stopped after 24 h by removal of the immobilized lipase. The determination of the composition of the reaction mixture as well as crystallization of 2-MG was performed as described previously (9). Purity of 2-MG was determined by gas chromatography (GC; Fisons Instruments Mega series, Mainz, Germany) using a nonpolar column (Optima 5, 25 m $\times$ 0.25 mm i.d.; Machery & Nagel, Düren, Germany) after derivatization of samples with *N*-methyl-*N*-trimethylsilylheptafluorobutyramide. Analysis was carried out using the following temperature program: 200°C for 2 min, then heating by 8°C/min to 360°C, and holding for 5 min. The purity of 2-MG given in Tables 1 and 2 was also confirmed by ^{13}C nuclear magnetic resonance spectroscopy.

In a similar manner, alcoholysis of fish oils I–III was performed, but reaction components were equilibrated to the water activities given in Table 7. Isolation of 2-monoglyc-

TABLE 2

Influence of Lipases on the Yield and Purity of 2-MP Obtained by Alcoholysis of Tripalmitin with Ethanol in MTBE (after 24 h)

Lipase from	Yield of 2-MP (%)	Purity of 2-MP (%)
<i>Rhizomucor miehei</i> ^a	88	80 ^c
<i>Rhizopus delemar</i> ^b	95	>95
<i>Rhizopus javanicus</i> ^b	50	>90 ^d

^aLipozyme immobilized on anion exchange resin.

^bImmobilized on Celite.

^c20% 1-MP.

^dContains 1-MP and dipalmitin. See Table 1 for abbreviations.

erides was performed by preparative HPLC or by column chromatography using boric acid-treated silica gel (11).

Esterification of 2-monopalmitin. This was performed in principle as described previously (9). Briefly, the purified 2-MP obtained from alcoholysis was esterified with oleic acid in *n*-hexane using Lipozyme or RDL immobilized on Celite at 38°C. All reaction components were pre-equilibrated to a water activity of 0.11. Activated molecular sieve was added to remove water generated during this step.

GC separation of triglycerides. The triglycerides obtained from the esterification of 2-MP were separated by GC analysis using the same equipment as described above with the following temperature program: 200°C followed by heating 8°C/min to 360°C, and holding for 20 min.

Regiospecific analysis of 1,3-oleoyl-2-palmitoyl-glycerol (OPO). This was performed by digestion of OPO with pancreatic lipase (12) followed by GC analysis.

RESULTS

Synthesis of OPO. Alcoholysis of tripalmitin. The first step in the synthesis of OPO was the alcoholysis of tripalmitin using *sn*-1,3-regiospecific lipases. Of three lipases [from *Rhizomucor miehei* (Lipozyme), RDL, and RJL] investigated, RDL immobilized on Celite gave the best yield (95% after crystallization) of 2-MP in methyl-*t*-butyl ether (MTBE) (Table 2). The alcoholysis reaction was very fast; after 8 h reaction time, tripalmitin was completely converted to 2-MP and only a small amount of 1,2 (2,3)-dipalmitin remained. After 24 h, the reaction mixture contained only 2-MP and palmitic acid ethyl ester and only a small amount of palmitic acid (Fig. 1). In contrast, a strong acyl migration took place when Lipozyme was used, because the monopalmitin fraction was composed of an 8:1 mixture of 2-MP/1-MP. We related this to the anion exchange resin serving as carrier for RML, which is known to promote acyl migration in MTBE (11). This acyl migration could be suppressed by using acetone as solvent; however, the yield of 2-MP (35%) was significantly lowered. In neat ethanol, no alcoholysis reaction with Lipozyme was observed (Table 1).

Yield and purity of 2-MP were also affected by the carrier material used for lipase immobilization. As shown in Figure 2, alcoholysis in MTBE was best performed using Lipozyme or RDL immobilized on Celite. Here, tripalmitin

TABLE 1
Influence of Organic Solvents on the Yield and Purity of 2-Monopalmitin (2-MP) Obtained by Alcoholysis of Tripalmitin with Lipozyme (after 24 h)

Organic solvent	Yield of 2-MP (%)	Purity of 2-MP (%)
MTBE	88	80 ^a
Acetone	35	>95
Ethanol	<1	n.d.

^a20% 1-MP; n.d., not detectable; MTBE, methyl-*t*-butyl ether.

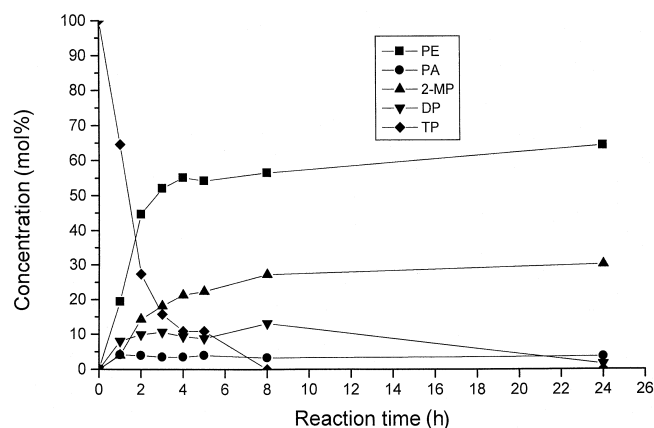


FIG. 1. Time course of the alcoholysis of tripalmitin with lipase from *Rhizopus delemar* immobilized on Celite. PE, palmitic acid ethyl ester; PA, palmitic acid; 2-MP, 2-monopalmitin; DP, dipalmitin; TP, tripalmitin; (mol%), mol of fatty acyl groups.

was completely consumed and mainly 2-MP was formed. Significantly lower yields of 2-MP and large concentrations of tripalmitin, dipalmitin, and palmitic acid remained when using RDL immobilized on polypropylene (EP 100) (Fig. 2).

Esterification of 2-MP with oleic acid. The second step in the synthesis of OPO is the esterification of 2-MP with oleic acid. The nutritional value of OPO greatly depends on the concentration of palmitic acid in the *sn*-2 position of the structured triglyceride. Even if highly pure 2-MP is used, acyl migration during esterification might lead to a reduction in the amount of palmitic acid in the *sn*-2 position. Water is known to promote acyl migration; thus, care must be taken that the water generated during esterification is quickly removed from the reaction mixture. This was achieved by using activated molecular sieves, *n*-hexane as solvent, and pre-equilibration of all reaction components to a low water activity of 0.11. Using these precautions, high yields of OPO (up to 72%) were obtained with Lipozyme or RDL immobilized on Celite (Table 3). Regiospecific analysis of the reaction prod-

TABLE 3

Synthesis of 1,3-Oleyl-2-palmitoyl-glycerol (OPO) by Esterification of 2-MP with Oleic Acid in *n*-Hexane with Immobilized Lipases (after 5 h)

Lipase from	Yield of OPO (%)	Positional distribution	
		1(3)-Position	2-Position
<i>Rhizomucor miehei</i> ^a	70	89% OA ^c	92% PA ^d
<i>Rhizopus delemar</i> ^b	72	83% OA ^c	94% PA ^d

^aLipozyme immobilized on anion exchange resin.

^bImmobilized on Celite.

^cOleic acid.

^dPalmitic acid. See Table 1 for abbreviation.

uct revealed that between 92 and 94% palmitic acid was located in the *sn*-2 position of OPO, while 83–89% oleic acid was bound to the *sn*-1 and *sn*-3 positions. Another reason for the low acyl migration observed during the second step might be the very short reaction time. After only 2 h the 2-MP was completely converted into OPO and only a minor amount of diglyceride was left (Fig. 3).

Alcoholysis of fish oil. In order to synthesize structured triglycerides containing PUFA like EPA and DHA, three different fish oils were subjected to an alcoholysis reaction. Initially, the fatty acid composition of the starting materials was determined by GC. The three fish oils differed considerably in their amount of PUFA. Fish oil I contained mainly palmitic acid and oleic acid, and the total PUFA content was 18.3%. Fish oil II was composed of 45.5% PUFA with DHA as the major component. Fish oil III lacked DHA, but contained 37.4% EPA (Table 4). In sharp contrast to the alcoholysis of tripalmitin, significantly lower yields of 2-MG were achieved in the alcoholysis of all fish oils, despite identical reaction conditions. The highest purity of 2-MP (>95%) was obtained using fish oil I, but only at 6.5% yield. Much higher yields (28–43%) were achieved with fish oils II and III, but here 1(3)-MG (25 and 50%) were formed too (Table 5). This was related to the difficult isolation of 2-MG. Crystallization was unsuccessful due to the low melting point of the PUFA, and

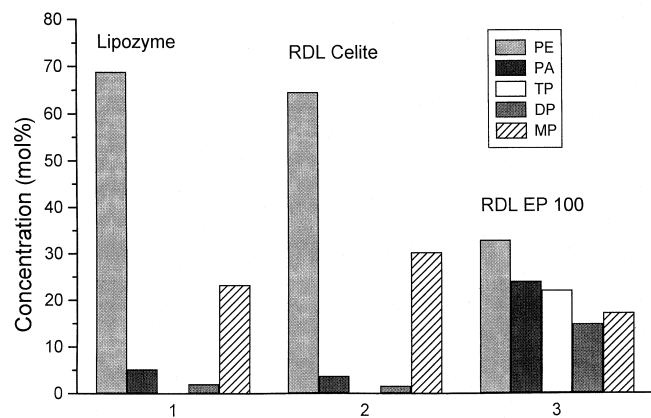


FIG. 2. Influence of carrier material for lipase immobilization on the yield of 2-MP in the alcoholysis of tripalmitin in methyl-*t*-butyl ether (after 24 h). RDL, lipase from *Rhizopus delemar*; RDL EP 100, RDL immobilized on polypropylene; Lipozyme, RML immobilized on anion exchange resin. For other abbreviations see Figure 1.

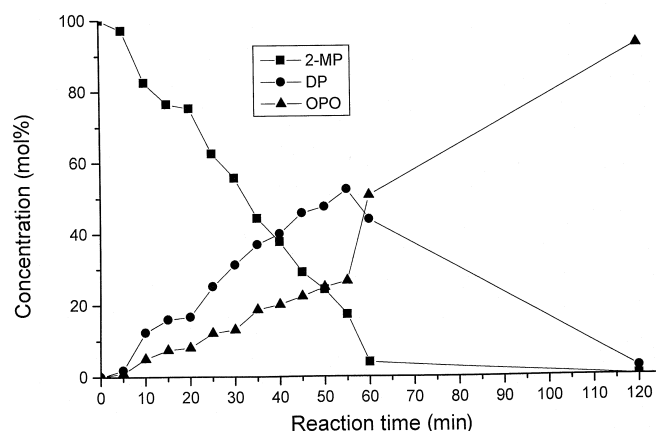


FIG. 3. Time course of the formation of 1,3-oleyl-2-palmitoyl-glycerol (OPO) from 2-MP with oleic acid in *n*-hexane with Lipozyme. For abbreviations, see Figure 1.

TABLE 4
Fatty Acid Composition of the Three Fish Oils
Used for the Lipase-Catalyzed Alcoholysis

Fatty acid	Fish oil I (mol%)	Fish oil II (mol%)	Fish oil III (mol%)
C _{14:0}	15.2	9.3	1.0
C _{16:0}	27.6	13.4	n.d. ^a
C _{16:1}	11.0	7.3	26.7
C _{18:0}	4.2	2.6	n.d.
C _{18:1}	17.0	9.8	4.9
C _{20:5}	10.5	11.4	37.4
C _{22:6}	7.8	34.1	n.d.

^an.d., not detectable.

TABLE 5
Synthesis of 2-Monoglycerides (2-MG) by Alcoholysis of Three Fish Oils with Ethanol in MTBE with Lipase from *Rhizopus delemar* Immobilized on Celite (after 24 h)

Fish oil	Yield of 2-MG (%)	Purity of 2-MG (%)
Fish oil I	6.5	>95
Fish oil II	28	75 ^a
Fish oil III	43	50 ^b

^aContains 25% 1-MG.

^bContains 50% 1-MG. See Table 1 for abbreviation.

the 2-MG fraction could only be isolated by preparative HPLC or silica gel column chromatography. Acyl migration observed during silica gel column chromatography could be suppressed by impregnation of silica gel with boric acid prior to the separation procedure (11).

An analysis of the fatty acid composition of the 2-MG clearly shows the enrichment of PUFA (Fig. 4). 2-MG derived from fish oil I contains more than 70% PUFA, with DHA as the major component. In the case of fish oil II, alcoholysis resulted in a significant enrichment of DHA, because the 2-MG was mainly composed of this fatty acid (>70%). 2-MG from fish oil III contained mainly EPA and palmitoleic

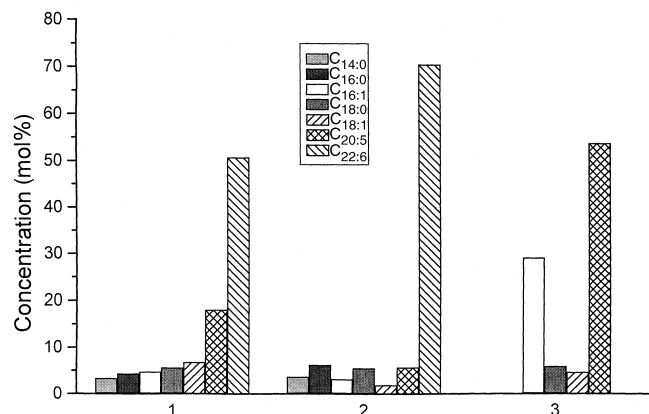


FIG. 4. Fatty acid composition of 2-monoglycerides obtained by alcoholysis of different fish oils in acetone using lipase from *Rhizopus delemar* immobilized on Celite. See Figure 1 for abbreviation.

TABLE 6
Influence of Lipases (all immobilized on Celite) on the Yield of 2-MG Obtained by Alcoholysis of Fish Oil I with Ethanol in Acetone (after 24 h)^a

Lipase from	Yield of 2-MG (%)
<i>Rhizopus delemar</i>	20
<i>Rhizopus javanicus</i>	19
<i>Rhizopus niveus</i>	14
<i>Candida rugosa</i>	5.5

^aSee Table 5 for abbreviation.

acid (C_{16:1}), the major components of the starting material (Fig. 4).

Yields of 2-MG in the alcoholysis of fish oil I could be further increased to 20% by using acetone as solvent (Table 6). Acetone has the advantage that it is allowed to be used in food and lowers acyl migration. Whereas other lipases from *Rhizopus* sp. were found suitable, lipase from *Candida rugosa* only led to the formation of 5.5% 2-MG from fish oil I. The strongest impact on 2-MG yield was observed by performing the alcoholysis reaction at different water activities (Table 7). Best results were achieved at a water activity of 0.94, at which 37 and 84% 2-MG were produced from fish oils I and III, respectively. In both cases immobilization of RDL on polypropylene (EP 100) using acetone as solvent was found most suitable.

DISCUSSION

We have shown in this paper that the two-step process allows the synthesis of 1,3-oleyl-2-palmitoyl-glycerol in high yield and purity. Furthermore, it was demonstrated that 2-MG containing PUFA can be prepared by alcoholysis of fish oils. In accordance with our previous papers (9,10)—and in contrast to direct synthesis by a one-step interesterification (6–8)—acyl migration can be suppressed in the alcoholysis reaction. However, in order to achieve best results, choice of solvent, carrier for lipase immobilization, and water activity must be carefully controlled and optimized for each new substrate. For instance, alcoholysis of tripalmitin is best performed with lipase from *Rhizopus delemar* immobilized on Celite in

TABLE 7
Influence of Solvent and Water Activity on the Yield of 2-MG Obtained by Alcoholysis of Fish Oils I and III with Ethanol (after 24 h)^a

Substrate	<i>Rhizopus delemar</i> immobilized on lipase		Water activity	Yield of 2-MG (%)
		Solvent		
Fish oil I	Celite	MTBE	0.43	6.5
	Celite	Acetone	0.43	20
	EP 100	Acetone	0.94	37
Fish oil III	Celite	MTBE	0.43	43
	EP 100	Acetone	0.94	84

^aSee Tables 1 and 5 for abbreviations.

MTBE (to achieve a high yield of 2-MP) or in acetone (to achieve a high purity of 2-MP). In contrast, in the alcoholysis of fish oil III, acetone was the best solvent and polypropylene was the best lipase support. Furthermore, the most appropriate water activity must always be determined too; it was 0.94 in the alcoholysis of fish oils I and III, but 0.11 in the alcoholysis of tripalmitin.

The isolation of 2-MG is easy to perform by crystallization as shown here for 2-MP and in previous papers for 2-monoolein and 2-monolinolein (9,10). 2-MG derived from fish oil could not be isolated by this manner, because the melting point of the monoglycerides is much too low. A separation by HPLC yields highly pure 2-MG, but this method is not practical on a large scale. We were able to isolate the 2-MG by column chromatography, but only after boric acid treatment of silica gel to avoid the formation of 1-MG. The alcoholysis of the three fish oils further allowed the enrichment of PUFA, because they comprised the major fatty acids present in the 2-MG fraction. It is expected that the formation of structured triglycerides from 2-MG containing PUFA can be performed in a similar manner to that shown in this paper for OPO and other structured triglycerides as described previously (9,10).

The esterification of 2-MP with oleic acid proceeded very fast, within 2 h, and almost no acyl migration occurred, as proven by positional analysis of the produced OPO. This contains 94% palmitic acid in *sn*-2 position and thus is much more pure compared to the currently marketed product Betapol, which contains only 65% palmitic acid in *sn*-2 position (13).

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