Lipase-Catalyzed Synthesis of Structured Triacylglycerides from 1,3-Diacylglycerides

Sirirung Wongsakul[a,b], Aran H-Kittikun[a], and Uwe T. Bornscheuer[b,*]

[a]Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112, Thailand, and [b]Institute of Chemistry and Biochemistry, Department of Technical Chemistry and Biotechnology, University of Greifswald, D-17487 Greifswald, Germany

ABSTRACT: A new method for the lipase-catalyzed synthesis of structured TAG (ST) is described. First, *sn*1,3-dilaurin or -dicaprylin were enzymatically synthesized using different published methods. Next, these were esterified at the *sn*2-position with oleic acid or its vinyl ester using different lipases. Key to successful enzymatic synthesis of ST was the choice of a lipase with appropriate FA specificity, i.e., one that does not act on the FA already present in the *sn*1,3-DAG, but that at the same time exhibits high selectivity and activity toward the FA to be introduced. Reactions were performed in the presence of organic solvents or in solvent-free systems under reduced pressure. With this strategy, mixed ST containing the desired compounds 1,3-dicapryloyl-2-oleyl-glycerol or 1,3-dilauroyl-2-oleyl-glycerol (CyOCy or LaOLa) were obtained at 87 and 78 mol% yield, respectively, using immobilized lipases from *Burkholderia cepacia* (Amano PS-D) in *n*-hexane at 60°C. However, regiospecific analysis with porcine pancreatic lipase indicated that in CyOCy, 25.7% caprylic acid and in LaOLa 11.1% lauric acid were located at the *sn*2-position. Oleic acid vinyl ester was a better acyl donor than oleic acid. Esterification of *sn*1,3-DAG and free oleic acid gave very low yield (<20%) of ST in a solvent system and moderate yield (>50%) in a solvent-free system under reduced pressure.

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KEY WORDS: *Burkholderia cepacia* lipase, *Candida antarctica* lipase B, 1,3-DAG, immobilized lipases, structured TAG.

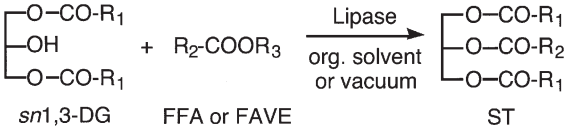
The nutritional value of TAG and their physicochemical properties not only are determined by the FA composition but also depend on the positional distribution of the acyl groups bonded to the glycerol. Structured TAG (ST) of the ABA-type containing medium-chain FA (MCFA; e.g., C_8) in *sn*1,3-positions and a long-chain unsaturated FA (e.g., C_{16}–C_{22}) in the *sn*2-position are an effective energy source for patients with malabsorption, e.g., pancreatic insufficiency. A range of methods for the enzymatic synthesis of these compounds have been already described (1,2). This includes acidolysis of a TAG (3,4) or interesterification between two TAG (5,6). Unfortunately, yields of ABA-type TAG are low, and a variety of by-products are formed. These are difficult to separate from the desired product. Significantly higher yields and purities can be achieved by a two-step process developed in our laboratories (7–9). In the first step, highly pure 2-MAG are obtained by alcoholysis of a TAG with ethanol using a 1,3-regiospecific lipase followed by crystallization. The 2-MAG thus obtained are subsequently esterified with a suitable FA in the second step to obtain the desired ST. More recently, we also reported the synthesis of ST containing PUFA from tuna oil in the *sn*2-position (10). However, crystallization of 2-MAG containing unsaturated FA is rather difficult due to the very low m.p. (i.e., –56°C for 2-docosahexaenoic acid glycerol ester).

An alternative would be the use of *sn*1,3-diacylglycerols (1,3-DAG) as starting materials, which are then esterified with a *sn*2-specific lipase (Scheme 1). A range of enzymatic methods for the synthesis of *sn*1,3-DAG in high yield and purity are described in the literature that use, e.g., glycerol adsorbed on silica gel and FA vinyl esters as acyl donors (11) or hydrolysis of TAG by *sn*1,3-specific lipases (12). 1,3-DAG obtained by lipase catalysis (13) have been introduced recently as alternative cooking and frying oils (14) and therefore are readily available on an industrial scale. Unfortunately, *sn*2-specific lipases are not available from nature, although some *sn*2-selectivity of a few lipases has been described (15,16). This was later attributed mostly to acyl migration and not to a positional selectivity of the enzyme (17).

We envisaged that the enzymatic synthesis of ST also should be feasible from 1,3-DAG by using a lipase with FA chain-length or FA saturation specificity. Accordingly, this enzyme must not act on the FA already present in the 1,3-DAG, but at the same time exhibit high selectivity and activity toward the FA to be introduced at the *sn*2-position. The investigation of this strategy is described in this paper.

EXPERIMENTAL PROCEDURES

Lipases. Lipases (TAG hydrolases, EC 3.1.1.3) were from *Rhizopus oryzae* [Amano D, D-EP100; immobilized on polypropylene (EP100) Amano Pharmaceuticals Co., Nagoya,



SCHEME 1

*To whom correspondence should be addressed at Institute of Chemistry and Biochemistry, Department of Technical Chemistry and Biotechnology, University of Greifswald, Soldmannstr. 16, D-17487 Greifswald, Germany.
E-mail: uwe.bornscheuer@uni-greifswald.de

Japan], *Burkholderia cepacia* (immobilized, Amano PS-D, formerly named *Pseudomonas cepacia*; Amano Pharmaceuticals Co.), *Rhizomucor miehei* (Lipozyme RM IM, Novozyme, Bagsvaerd, Denmark), lipase B from *Candida antarctica* (CAL-B; Novozyme), and porcine pancreatic lipase (Amano Pharmaceuticals Co.). All chemicals and solvents used were reagent grade and purchased from common commercial suppliers, with the exception of EP 100 (particle size 200–400 μm; Akzo, Obernburg, Germany).

Synthesis of 1,3-DAG by esterification of glycerol and FFA or FA vinyl esters (FAVE). 1,3-DAG was synthesized from glycerol (immobilized on silica gel) and FFA or FAVE in methyl-*tert*-butyl ether (MTBE) at room temperature catalyzed by Lipozyme RM IM as described (11).

1,3-DAG were also synthesized by esterification of glycerol with FFA or FAVE in a solvent-free system or in an organic solvent catalyzed by CAL-B according to a published method (12) with minor modifications. Activated molecular sieves were added or vacuum was applied in reactions with FFA to remove water produced during the reaction. The 1,3-DAG were recovered and purified by crystallization in *n*-hexane at –20°C. If high concentrations of 2-MAG were present, recrystallization in dry methanol at –20°C was performed to obtain purer 1,3-DAG. Samples from the reaction mixture were periodically withdrawn to determine the acylglycerol composition by TLC-FID (see below).

Alcoholysis of TAG for the synthesis of 1,3-DAG. This reaction was carried out using a modified method for 2-MAG synthesis (10). The substrate mixture was pre-equilibrated to a water activity of a_w 0.11 for 48 h before use. The TAG/ethanol ratio was 1:1 to 1:2 (vol/vol). Samples from the reaction mixture were periodically withdrawn and diluted with chloroform, followed by the determination of acylglycerol composition by TLC-FID (see below). After 24 h, the immobilized lipase was separated from the reaction mixture by centrifugation to stop the reaction.

Synthesis of ST from 1,3-DAG and FFA/FAVE. ST were synthesized by esterification of 1,3-DAG and oleic acid (OA) or oleic acid vinyl ester (OAVE) in *n*-hexane at 60°C using PS-D as biocatalyst. 1,3-DAG (0.1 mmol) and OA or OAVE (0.2 mmol) were dissolved in 1 mL *n*-hexane in 2-mL screw-capped vials. Activated molecular sieves were added, or vacuum was applied when OA was used as acyl donor, for water removal. The reaction was started by addition of PS-D (10% w/w of 1,3-DAG). Samples from the reaction mixture were periodically withdrawn for the determination of acylglycerol composition by TLC-FID and HPLC.

Purification of produced TAG. Lipase was removed by centrifugation, and excess solvent was removed by a nitrogen stream. The reaction mixture was dissolved in *n*-hexane and purified by silica gel column chromatography. Elution was performed with a gradient starting from *n*-hexane/diethylether (99:1) and subsequent increase of diethylether concentration up to pure diethylether. The composition of each fraction was determined separately by TLC-FID.

Analytical methods. Changes in acylglycerol composition during the reaction were quantitatively determined by TLC-FID analysis (Iatroscan; Iatron Laboratories, Tokyo, Japan) (18). The FA compositions of acylglycerols were determined by converting all FA into the corresponding FAME followed by GC analysis as described previously (10). For ST analysis, the reaction mixture was separated by TLC using a solvent system of chloroform/methanol/ammonium (70:0.4:0.1 by vol) and visualized by iodine vapor. The band corresponding to TAG was scratched-off, followed by methylation for GC analysis.

HPLC separation of TAG. The composition of the TAG formed during the enzymatic esterification was determined by HPLC using a Nucleosil ODS column, (5 μm, 250 × 4.6 mm; Agilent, Waldbronn, Germany) and an ELSD (Polymer Labs, Darmstadt, Germany) at a flow rate of 1.5 mL/min. Elution was performed using a gradient elution system of acetonitrile and dichloromethane (70 to 55% acetonitrile over 10 min, followed by 55 to 70% acetonitrile over 8 min).

Regiospecific analysis of TAG. The distribution of the FA on the TAG was determined by digestion of TAG with porcine pancreatic lipase followed by GC analysis (9). To a 5-mL screw-capped tube, 20 mg of purified TAG, 10 mg pancreatic lipase, and 3 mL Tris-HCl buffer (pH 8, 1 M) were added. After shaking, 0.5 mL sodium deoxycholate solution (1 g/L) and 0.2 mL calcium chloride solution (220 g/L) were added. After vigorous shaking, the tube was immediately placed in a water bath (40°C) for 1 min. Then the tube was shaken for 2 min and cooled in running water before 1 mL hydrochloric acid (6 M) and 1 mL diethylether were added, followed by vigorous shaking for 1 min. After separation of the phases by centrifugation, the components of the resulting mixture of partial acylglycerols in the ether phase were separated by preparative TLC using acetone/chloroform (96:4 by vol) as developing solvent and visualized by iodine vapor. The 2-MAG band was scraped off, methylated, and analyzed by GC.

RESULTS AND DISCUSSION

Enzymatic synthesis of 1,3-DAG. The highest 1,3-DAG yields [93% for 1,3-dicaprylin (1,3-DCy) 73% for 1,3-dilaurin, (1,3-DLa)] were obtained after 4–6 h in the esterification between FAVE and glycerol at 0°C in a solvent-free system using CAL-B as biocatalyst. The molar ratio of 1,3-DAG and FAVE was 1:2 (mol/mol). The yield thus obtained was higher than the one found in the esterification of glycerol adsorbed on silical gel (11) (75% 1,3-DCy; 65% 1,3-DLa). Direct esterification of FFA and glycerol was slow and gave only moderate yields (60% 1,3-DCy; 60% 1,3-DLa) with high amounts of MAG present. However, the yield could be raised with increasing temperature and under reduced pressure (data not shown).

Enzymatic synthesis of ST from 1,3-DAG. As pointed out in the introduction, the synthesis of an ST from *sn*1,3-DAG and a *sn*2-destined FA should be possible with a lipase having a well-defined FA specificity. To demonstrate the practicability of this new strategy, the nonregiospecific lipase PS-D was chosen, as it preferably acts on long-chain FA (LCFA) rather than MCFA (Wongsakul, S., unpublished observations). Thus, using a 1,3-

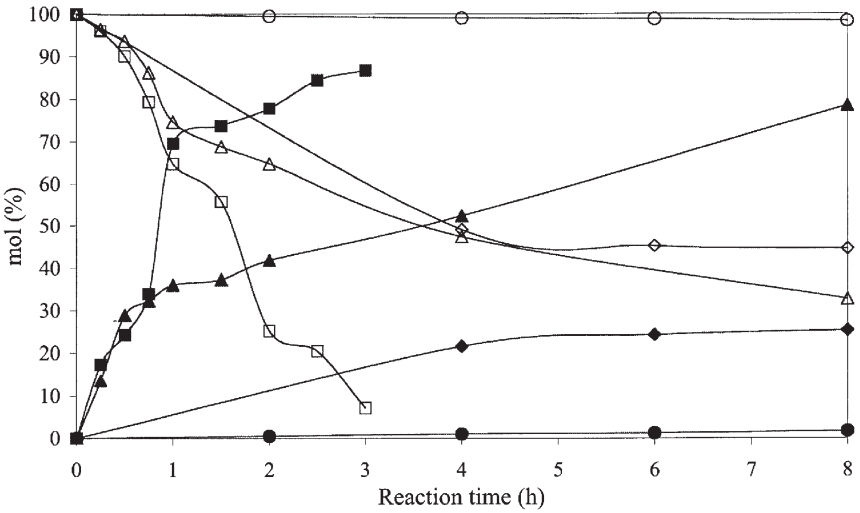


FIG. 1. Time course of the esterification between 1,3-DAG and oleic acid vinyl ester or oleic acid in *n*-hexane catalyzed by lipase Amano PS-D at 60°C (small-scale synthesis). TAG from 1,3-DCy + OAVE (■), TAG from 1,3-DCy + OA (●), DAG decrease from 1,3-DCy + OAVE (□), DAG decrease from 1,3-DCy + OA (○), TAG from 1,3-DLa + OAVE (▲), TAG from 1,3-DLa + OA (◆), DAG decrease from 1,3-DLa + OAVE (△), DAG decrease from 1,3-DCy + OA (◇); 1,3-DCy, 1-3-dicaprylin; OAVE, oleic acid vinyl ester; OA, oleic acid; 1,3-DLa, 1,3-dilaurin.

DAG composed of MCFA and a LCFA as acyl donor, ST synthesis should be feasible. Indeed, esterification of 1,3-dicaprylin or -dilaurin with OAVE in *n*-hexane resulted in high yields of TAG (87 and 78%, respectively) at very short reaction times (3 and 8 h) (Fig. 1). GC analysis of these ST showed that the ratio of MCFA to LCFA was about 2:1 (Table 1). Esterification of 1,3-DCy with OAVE performed at gram-scale was finished after only 2 h (Fig. 2). HPLC analysis of the product mixture revealed that the desired ST CyOCy (59.4%) was most prominent followed by CyOO (32.8%). Triolein was formed in only very small amounts (0.65%). It should be noted that this method does not allow distinction between regioisomers. Thus, the TAG were purified and partially degraded using porcine pancreatic lipase, followed by GC analysis of the 2-MAG fraction. The result showed that CyOCy/CyOO contained 74.3% OA and 25.7% caprylic acid at the *sn*2-position, whereas LaOLa/ LaOO contained 88.9% OA and 11.1% lauric acid at the *sn*2-position.

When free OA was used as acyl donor, water produced during the esterification was removed either by applying vacuum (solvent-free system) or by adding molecular sieve (solvent system), to avoid acyl migration and an unfavorable equilibrium. Esterification between 1,3-DAG and OA in *n*-hexane was very slow, and only 20% TAG was obtained after 24 h in the esterification starting from 1,3-dilaurin, and almost no reaction occurred using 1,3-dicaprylin (Fig. 1). Yields and reaction rates were considerably increased to 56% (from 1,3-dicaprylin) and 53% (from 1,3-dilaurin) in the solvent-free system under reduced pressure (Fig. 3).

TABLE 1
FA Composition of Structured TAG Products

	FA (%)[a]		
Structured TAG	C8:0	C12:0	C18:1
CyOCy[b]	61.0	—	39.0
LaOLa[b]	—	63.3	36.7

[a]As determined by GC analysis
[b]CyOCy, structured TAG obtained from esterification of 1,3-dicaprylin and oleic acid vinylester; LaOLa, structured TAG obtained from esterification of 1,3-dilaurin and oleic acid vinylester. Note: The data also include regioisomers

A new enzymatic method for the synthesis of ST starting from 1,3-DAG and a FA (or its vinyl ester) was developed. The yields are comparable to the previously described two-step method (7–9), which is based on TAG alcoholysis to yield 2-MAG followed by esterification at the *sn*1,3-positions. However, in this method, the yield of the desired ST (CyOCy or LaOLa) is lower due to the presence of other TAG species. In addition, the regioisomers (i.e., CyCyO or LaLaO) were found after analysis using PPL degradation. However, it cannot be excluded that partial acyl migration occurred during this analytical method. Further improvement of the reaction conditions is under study to avoid the formation of undesired ST and to apply alternative methods for regiospecific analysis.

Still, several major advantages of this new method exist, which include the easier synthesis of regioisomerically pure *sn*1,3-DAG. These are much more stable than the *sn*2-MAG, obtained from alcoholysis of TAG, which can easily undergo acyl migration to yield *sn*1(3)-MAG. We assume that it will also be easier to produce ST bearing PUFA such as EPA or DHA at the *sn*2-position, as they are difficult to obtain using the two-step method owing to difficulties in the isolation of their *sn*2-MAG. The alternative would be an acidolysis reaction between, e.g., fish oil and another FA, but this results in low yields, a mixture of a broad range of TAG species and acyl migration can occur. In contrast, it has been demonstrated here that lipase from *B. cepacia* (Amano PS-D) efficiently introduced OA to the *sn*2-position of two 1,3-DAG

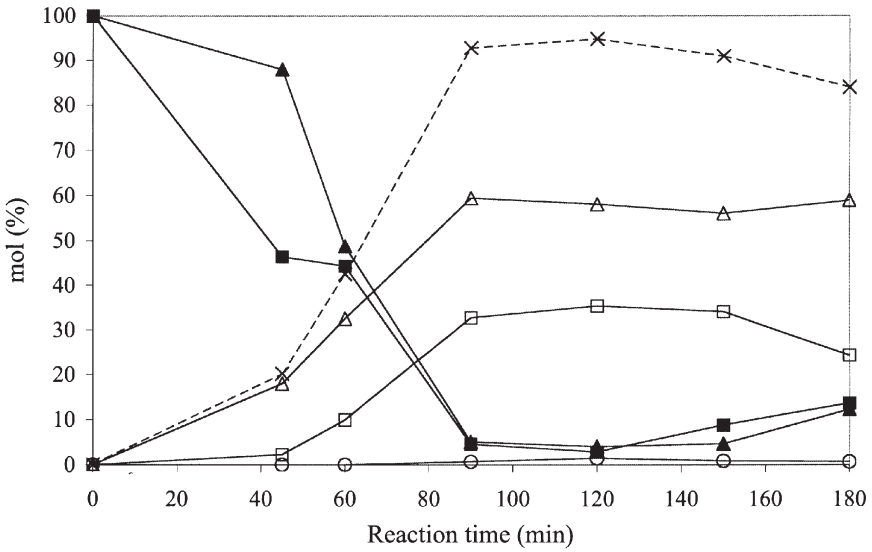


FIG. 2. Time course of the esterification between 1,3-DCy and OAVE in *n*-hexane catalyzed by lipase Amano PS-D at 60°C (gram-scale synthesis). 1,3-DCy (▲), OAVE (■), CyOCy (△), CyOO (□) , triolein (○) , total TAG (×). CyOCy, 1,3-dicapryloyl-2-oleyl-glycerol; CyOO, 1-capryloyl-2,3-dioleyl-glycerol; for other abbreviations see Figure 1.

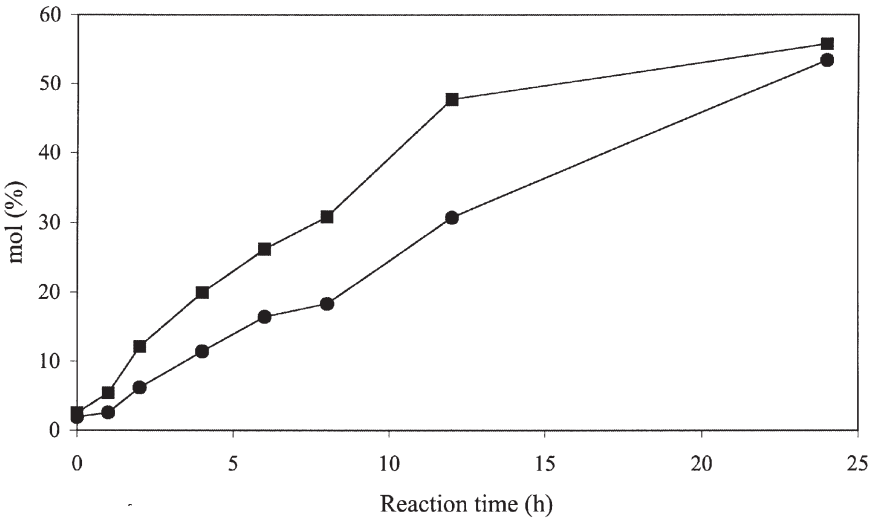


FIG. 3. Structured triacylglycerol production by esterification of 1,3-DCy (■) or 1,3-DLa (●) with OA in a solvent-free system catalyzed by Amano PS-D at 60°C under reduced pressure. For abbreviations see Figure 1.

serving as model compounds and rarely acted on the MCFA present at the *sn*1- or 3-positions.

Although enzymatic esterification of glycerol or 1,3-DAG with a FAVE proceeded faster and gave higher yields of 1,3-DAG or ST in the solvent-free system, cheaper FFA might be the preferred starting material for large-scale synthesis. This approach is also under current investigation.

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