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Solvent-free Lipase-Catalyzed Preparation of Diacylglycerols

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Various methods have been applied for the enzymatic preparation of diacylglycerols that are used as dietary oils for weight reduction in obesity and related disorders. Interesterification of rapeseed oil triacylglycerols with commercial preparations of monoacylglycerols, such as Monomuls 90-O18, Mulgaprime 90, and Nutrisoft 55, catalyzed by immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) in vacuo at 60 °C led to extensive (from 60 to 75%) formation of diacylglycerols. Esterification of rapeseed oil fatty acids with Nutrisoft, catalyzed by Lipozyme RM in vacuo at 60 °C, also led to extensive (from 60 to 70%) formation of diacylglycerols. Esterification of rapeseed oil fatty acids with glycerol in vacuo at 60 °C, catalyzed by Lipozyme RM and lipases from *Thermomyces lanuginosus* (Lipozyme TL IM) and *Candida antarctica* (lipase B, Novozym 435), also provided diacylglycerols, however, to a lower extent (40−45%). Glycerolysis of rapeseed oil triacylglycerols with glycerol in vacuo at 60 °C, catalyzed by Lipozyme TL and Novozym 435, led to diacylglycerols to the extent of ≤50%. Repeated use of Lipozyme RM in the esterification of rapeseed oil fatty acids with Monomuls resulted in minor reduction of its activity. The products of esterification of rapeseed oil fatty acids with Monomuls and glycerol yielded upon short-path vacuum distillation residues (diacylglycerol oils) containing 66−70% diacylglycerols.

KEYWORDS: Diacylglycerols; monoacylglycerols; triacylglycerols; lipases; esterification; transesterification; glycerolysis; rapeseed oil; rapeseed oil fatty acids

INTRODUCTION

Diacylglycerols (DAG) are naturally occurring minor constituents of edible fats and oils that mainly consist of triacylglycerols. Moreover, DAG are formed by hydrolysis of triacylglycerols in the intestinal tract, which is catalyzed by pancreatic lipase. Mixtures of mono- and diacylglycerols (mono- and diglycerides) are important emulsifiers that are widely used in industrially processed foods. Recently, it has been found that DAG, particularly *sn*-1,3-diacylglycerols, may have beneficial effects with regard to the prevention of obesity (*1*−*6*) and lipemia (*7*−*9*), despite having a similar energy value and digestibility as known for triacylglycerols (*10*). In Japan DAG have been designated "Food for Specified Health Use" (FOSHU) since 1999, and a cooking oil containing ∼80% DAG (DAG oil) as well as mayonnaise are on the market (*11*, *12*). In the United States DAG oil has received the status "Generally Recognized as Safe" (GRAS) by the U.S. Food and Drug Adminstration (*11*).

Several methods are available for the preparation of DAG. Chemical methods are recommended for the preparation of individual, stereochemically pure DAG, such as *sn*-1,2-diacylglycerols, *sn*-2,3-diacylglycerols, and *sn*-1,3-diacylglycerols. A lipase-catalyzed preparation of DAG and monoacylglycerols by partial hydrolysis of triacylglycerols of fats and oils and the esterification of fatty acids to glycerol and interesterification of triacylglycerols with glycerol (glycerolysis) are also known (*13*, *14*). Lately, the synthesis of mono- and dioleoylglycerol by lipase-catalyzed glycerolysis of trioleoylglycerol has been reported (*15*, *16*). For the preparation of DAG oil for food use common and highly unsaturated fatty acids have been esterified to glycerol, catalyzed by lipase from *Rhizomucor miehei* (*11*, *17*, *18*). Little is known, however, about the production of DAG for nutritional purposes by lipase-catalyzed esterification or transesterification of monoacylglycerols and glycerol with rapeseed oil fatty acids or rapeseed oil in solvent-free systems under vacuum that we describe in the present paper. Such an approach has been compared with lipase-catalyzed esterification of fatty acids with glycerol and glycerolysis of triacylglycerols with glycerol.

MATERIALS AND METHODS

Chemicals. Commercial mixtures of monoacylglycerols were gifts from Cognis Deutschland GmbH, Düsseldorf, Germany (Monomuls 90-O18), Cognis Grünau Illertissen GmbH, Illertissen, Germany (Nutrisoft 55), and Mühlenchemie GmbH, Ahrensburg, Germany (Mulgaprime 90). Glycerol, silica gel H, and silica gel 60 as well as 1-methylimidazole were products of Merck-VWR International (Darmstadt, Germany). Trimethylsulfonium hydroxide (TMSH) reagent and *N*-methyl-*N*-trimethylsilylheptafluorobutyramide (MSHFBA) were purchased from Macherey-Nagel (Düren, Germany). The immobilized lipase preparations from *R. miehei* (Lipozyme RM IM), *Candida*

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Table 1. Methods Used for the Preparation of the Diacylglycerols

reactants		lipase	reaction conditions	reaction type
rapeseed oil triacylglycerols (0.1 mmol, 88 mg)	Monomuls 90-O18 (0.3 mmol, 106 mg)	Lipozyme RM IM (25 mg, 13 wt % of substrates)	vacuum (20–40 hPa); 50, 60, 80 °C	interesterification
rapeseed oil triacylglycerols (0.1 mmol, 88 mg)	Mulgaprime 90 (0.3 mmol, 102 mg)	Lipozyme RM IM (25 mg, 13 wt % of substrates)	vacuum (20–40 hPa); 50, 60, 80 °C	interesterification
rapeseed oil fatty acids (1 mmol, 283 mg)	Nutrisoft 55 (1 mmol, 340 mg)	Lipozyme RM IM (57 mg, 9 wt % of substrates)	vacuum (20–40 hPa); 60, 80 °C	esterification
rapeseed oil fatty acids (3 mmol, 849 mg)	glycerol (1 mmol, 92 mg) on silica gel (180 mg)	Lipozyme RM IM, Lipozyme TL IM, Novozym 435, 84 mg, 9 wt % of substrates, each	vacuum (20–40 hPa); 60 °C	esterification
rapeseed oil triacylglycerols (0.5 mmol, 443 mg)	glycerol (0.5 mmol, 46 mg) on silica gel (90 mg)	Lipozyme TL, Novozym 435, or *C. rugosa*, 42 mg, 9 wt % of substrates, each	vacuum (20–40 hPa); 60 °C	interesterification (glycerolysis)
rapeseed oil triacylglycerols (0.5 mmol, 443 mg)	glycerol (1 mmol, 92 mg) + water (0.5 mmol, 9 mg)	Lipozyme RM IM, Lipozyme TL IM, Novozym 435, or *C. rugosa*, 42 mg, 8 wt % of substrates, each	vacuum (20–40 hPa); 60 °C	interesterification (glycerolysis)

antarctica (lipase B, Novozym 435), and *Thermomyces lanuginosus* (Lipozyme TL IM) were kindly provided by Novozyme, Bagsvaerd, Denmark. Lipase (type VII) from *Candida rugosa* and 1-octacosanol were obtained from Sigma-Aldrich (Deisenhofen, Germany). Rapeseed oil "Holstensegen" (Brökelmann and Co., Hamm, Germany) was purchased from a local supermarket. Fatty acids were prepared from rapeseed oil via saponification with potassium hydroxide, followed by treatment of the resulting soaps with hydrochloric acid. Glycerol was adsorbed on silica gel 60 (particle size = 0.063−0.2 mm) in a ratio of 1:2 (w/w) by intensive mixing until the final product was a free-flowing powder.

Lipase-Catalyzed Reactions. Commercial monoacylglycerols were transesterified with rapeseed oil triacylglycerols or esterified with fatty acids derived from rapeseed oil in the presence of various immobilized lipase preparations by magnetic stirring in a screw-capped tube in vacuo (20−40 hPa, measured at room temperature) at 60 or 70 °C for various periods. Water was trapped in the gas phase using KOH pellets, which were placed within plugs of glass wool in the outlet tube connecting the reaction vessel with vacuum. Similarly, glycerol was transesterified with rapeseed oil triacylglycerols or esterified with fatty acids derived from rapeseed oil in the presence of various lipase preparations in vacuo.

In one set of experiments, esterifications of Monomuls 90-O18 monoacylglycerols (2 mmol, 706 mg) were carried out with rapeseed oil fatty acids (2 mmol, 562 mg), catalyzed by *R. miehei* lipase (Lipozyme RM IM, 60 mg, 4.7 wt % of enzyme based on weight of substrates) in vacuo (20 hPa) at 60 °C for 2 h. Thereafter, the biocatalyst was removed by filtration and used repeatedly for nine consecutive reactions under identical conditions using fresh substrate mixture each time.

Lipid Extraction. Aliquots of total lipids were withdrawn from the reaction mixtures, dissolved in dichloromethane, filtered through a 0.45 μm syringe filter, and concentrated in a stream of nitrogen at 30−40 °C before lipid analysis and derivatization.

Lipid Analysis. *Thin-Layer Chromatography (TLC).* Aliquots of total lipids were withdrawn from the reaction mixtures at different times, and the conversion was checked by TLC on 0.3 mm layers of silica gel H. The plates were predeveloped with diethyl ether (∼4 cm) and then developed in isohexane/diethyl ether/acetic acid (60:40:1, v/v); spots were located by iodine staining. Alternatively, the TLC plates were sprayed with 30% aqueous sulfuric acid and heated in an oven kept at 200 °C.

Similarly, aliquots of total lipids were fractionated by chromatography on 0.5 mm layers of silica gel 60 G impregnated with boric acid (5 g of boric acid/100 g of silica gel). The plates were predeveloped with diethyl ether (∼4 cm) and then developed in isohexane/diethyl ether (3:2, v/v). The following fractions were isolated for fatty acid analyses: triacylglycerols, *rac*-1(3),2-diacylglycerols, 1,3-diacylglycerols, monoacylglycerols, and unesterified fatty acids.

Preparation of Derivatives for Gas Chromatography (GC). The fractions of tri-, di-, and monoacylglycerols as well as unesterified fatty acids that had been identified by cochromatography with standards were scraped out of the boric acid impregnated silica gel layers and extracted from silica gel with a mixture of methanol and water-saturated diethyl ether (1:1, v/v) followed by water-saturated diethyl ether. The combined extracts were filtered through a 0.45 μm syringe filter, blown with nitrogen until dry, and stored in isohexane under nitrogen at −20 °C. Aliquots of unesterified fatty acids were converted to the corresponding methyl esters by treatment with diazomethane. Aliquots of the other lipid fractions (∼2 mg, each) were dissolved in 40 μL of methyl *tert*-butyl ether and treated with 20 μL of trimethylsulfonium hydroxide to prepare methyl esters of their constituent fatty acids. The mixtures were shaken vigorously and kept at 75 °C for 15 min prior to direct injection onto a gas chromatograph.

Aliquots of total lipids (∼5−20 mg) that had been treated with diazomethane were silylated for high-temperature gas chromatography (HT-GC) using 100 μL of MSHFBA reagent in the presence of 5 μL of 1-methylimidazole at 110 °C for 30−60 min. After cooling, the reagents were removed in a stream of nitrogen, and the residual mixture was dissolved in dichloromethane for GC injection.

Gas Chromatography. Fatty acid methyl esters were analyzed by GC in a Hewlett-Packard 5890 series II instrument (Böblingen, Germany) on a 40 m DB-23 (methyl/50% cyanopropyl silicone, J&W, ASS-Chem, Bad Homburg, Germany), fused silica capillary column (0.18 mm i.d., 0.2 μm film thickness). Hydrogen was used as carrier gas (column head pressure = 142 kPa) at a split ratio of 1:10. Temperature was programmed from 160 °C, 2 min, at 1 °C/min to 178 °C, then at 8 °C/min to 225 °C, 2 min, and finally at 10 °C/min to 250 °C, 10 min. Injector and flame ionization detector (FID) temperatures were set at 280 °C. Peaks were integrated using Hewlett-Packard GC ChemStation software.

The fractions of tri-, di-, and monoacylglycerols were also analyzed by HT-GC in an Agilent 6890 series GC system PLUS or a Hewlett-Packard 5890 series II instrument (Böblingen, Germany) on a 12 m HT5 AQ (SGE, Darmstadt, Germany) fused silica capillary column (0.22 mm i.d., 0.1 μm film thickness). Hydrogen was used as carrier gas (column head pressure = 80 kPa) at a split ratio of 1:10. Temperature was programmed from 100 °C, 2 min, at 10 °C/min to 420 °C, 3 min. Injector and FID temperatures were set at 420 °C. Peaks were integrated as described above. Response factors of FID were determined, for example, for tri-, di-, and monoacylglycerols as well as fatty acid methyl esters, using purified compounds and 1-octacosanol as an internal standard. All GC samples, dissolved in isohexane or dichloromethane, were filtered through a 0.45 μm syringe filter before injection onto the gas chromatograph.

Vacuum Distillation. Short-path vacuum distillation was carried out in a 25 mL round-bottom flask under the following conditions: heating oil temperature, 180 °C for 30 min followed by 200 °C for 90 min; pump vacuum, 10^{-3} mbar. Reaction mixtures used were those resulting from transesterification of 1 mmol of either Mulgaprime (342 mg) or Monomuls (353 mg) with 1 mmol (887 mg) of rapeseed oil triacyl-

Table 2. Fatty Acid Composition of Commercial Monoacylglycerols as Well as Rapeseed Oil and Rapeseed Oil Fatty Acids Derived Therefrom

commercial monoacylglycerol or rapeseed oil	fatty acid composition[a] (wt %)					
	16:0	18:0	18:1ω9	18:2ω6	18:3ω3	others[b]
Mulgaprime[c]	47.0	46.7	tr[h]	tr	nd[i]	5.9
Monomuls[d]	9.6	3.4	76.4	9.2	nd	1.4
Nutrisoft[e]	56.4	13.9	23.0	4.5	nd	2.2
rapeseed oil[f,g]	4.4	1.7	60.0	18.9	7.6	7.4

[a] Fatty acids are designated by number of carbon atoms: number of double bonds; ωx indicates the position of the first double bond counted from the methyl end. [b] Other acyl moieties including 12:0, 14:0, 16:1, 20:0, 20:1, 22:0, 22:1. [c] Mulgaprime 90, Mühlenchemie GmbH, Ahrensburg (Germany). [d] Monomuls 90-O18, Cognis Deutschland GmbH, Düsseldorf (Germany). [e] Nutrisoft 55, Cognis Grünau Illertissen GmbH, Illertissen (Germany). [f] Holstensegen pure refined vegetable oil, Brökelmann + Co., Hamm (Germany), consisting of >95% triacylglycerols. [g] Rapeseed oil or fatty acids derived therefrom. [h] tr = trace (<0.5%). [i] nd, not detected.

glycerols and esterification of 2 mmol (184 mg) of glycerol/360 mg of silica gel and 4 mmol (1132 mg) of rapeseed oil fatty acids. Two lots of products resulting from each of the above reactions were pooled and used for distillation.

RESULTS AND DISCUSSION

Methods of Reaction and Composition of Starting Materials. Diacylglycerols were prepared by lipase-catalyzed reactions in different ways using commercial monoacylglycerols or glycerol as the starting materials. Methods used for the preparation of the diacylglycerols are outlined in **Table 1**.

Table 2 shows the fatty acid composition of commercial monoacylglycerols as well as rapeseed oil and rapeseed oil fatty acids derived therefrom. These results show that Mulgaprime monoacylglycerols mainly consist of saturated fatty acids (~86% of palmitic and stearic acids), whereas the constituent fatty acids of Monomuls monoacylglycerols are predominantly unsaturated (~85% of oleic and linoleic acids). Nutrisoft monoacylglycerols are characterized by high proportions of saturated fatty acids (~70%) and moderate proportions of unsaturated fatty acids (~28%). The compositions of zero-erucic rapeseed oil and the fatty acids derived therefrom are similar to those well-known from the literature.

Transesterification of Monoacylglycerols with Rapeseed Oil Fatty Acids. Table 3 shows the composition of the constituent fatty acids of various lipid fractions after Lipozyme RM IM-catalyzed transesterification of Monomuls 90-O18 monoacylglycerols with rapeseed oil triacylglycerols (**Table 1**) for 15 and 120 min. Apparently, the acyl composition of the various reaction products such as mono- and diacylglycerols did not change appreciably between 15 and 120 min, which may be attributed to the similar fatty acid composition of both starting materials, that is, Monomuls monoacylglycerols and rapeseed oil as shown in **Table 2**.

The results given in **Table 4** show the composition of constituent fatty acids of various lipid fractions after Lipozyme RM IM-catalyzed transesterification of Mulgaprime 90 monoacylglycerols with rapeseed oil triacylglycerols (**Table 1**) for 15 and 120 min. The two starting materials, that is, Mulgaprime monoacylglycerols and rapeseed oil, had distinctly different fatty acid compositions (**Table 2**). Thus, it is evident from the data given in **Table 4** that lipase-catalyzed transesterification leads to extensive incorporation of rapeseed oil fatty acids, especially oleic and linoleic acids, into mono-, di-, and triacylglycerol fractions.

Table 3. Composition of Constituent Fatty Acids of Various Lipid Fractions after Lipozyme RM IM-Catalyzed (13 wt % Enzyme Based on Weight of Substrates) Transesterification of Monomuls 90-O18 Monoacylglycerols (0.3 mmol, 106 mg) with Rapeseed Oil Triacylglycerols (0.1 mmol, 88 mg) at 60 °C in Vacuo (20 hPa) for 15 and 120 min

fatty acid[a]	time (min)	fatty acid composition (wt %) of lipid fractions[b]				
		MAG	1,2(2,3)-DAG	1,3-DAG	FA	TAG
16:0	15	4.5	4.5	4.9	6.6	5.0
	120	5.8	5.2	5.5	9.2	5.3
18:0	15	4.1	3.2	3.9	4.9	2.2
	120	4.2	3.6	3.9	6.9	2.7
18:1ω9	15	79.0	70.5	79.9	74.5	63.6
	120	75.9	71.2	78.6	71.4	69.9
18:1ω7	15	1.2	1.6	1.6	2.1	3.3
	120	1.9	1.8	2.0	2.1	3.2
18:2ω6	15	10.4	15.9	9.2	10.8	19.0
	120	10.5	15.2	8.9	9.1	14.7
18:3ω3	15	0.8	4.3	0.5	1.1	6.9
	120	1.6	3.0	1.1	1.3	4.2

[a] Fatty acids are designated as described in **Table 2**. [b] Abbreviations: 1,2(2,3)-DAG, 1,2(2,3)-diacylglycerols; 1,3-DAG, 1,3-diacylglycerols; FA, unesterified fatty acids; MAG, monoacylglycerols; TAG, triacylglycerols.

Table 4. Composition of Constituent Fatty Acids of Various Lipid Fractions after Lipozyme RM IM-Catalyzed (16 wt % Enzyme Based on Weight of Substrates) Transesterification of Mulgaprime 90 Monoacylglycerols (0.1 mmol, 34 mg) with Rapeseed Oil Triacylglycerols (0.14 mmol, 124 mg) at 60 °C in Vacuo (20 hPa) for 15 and 120 min

fatty acid[a]	time (min)	fatty acid composition (wt %) of lipid fractions[b]				
		MAG	1,2(2,3)-DAG	1,3-DAG	FA	TAG
16:0	15	30.2	17.1	28.1	18.1	11.0
	120	23.2	17.8	20.7	33.4	16.4
16:1ω7 + ω9	15	1.3	nd[c]	0.5	1.0	0.3
	120	1.8	1.3	0.3	4.2	nd
18:0	15	25.7	13.4	26.9	11.8	8.0
	120	17.5	14.2	20.1	17.9	15.1
18:1ω9	15	31.7	43.6	32.9	52.5	53.9
	120	42.4	44.8	44.6	30.7	47.3
18:1ω7	15	1.8	2.0	2.3	3.7	3.0
	120	2.6	2.2	3.0	1.6	2.5
18:2ω6	15	7.0	17.5	7.1	10.4	17.4
	120	9.9	14.5	8.9	8.3	14.2
18:3ω3	15	2.3	6.4	2.2	2.5	6.4
	120	2.6	5.2	2.4	3.9	4.5

[a,b] As given in **Table 2**. [c] nd, not detected.

Transesterification of Monoacylglycerols with Rapeseed Oil Triacylglycerols. Figure 1 shows the time course of the formation of diacylglycerols via transesterification of 300 μmol of Monomuls 90-018 monoacylglycerols with 100 μmol of rapeseed oil triacylglycerols catalyzed by 25 mg of immobilized *R. miehei* lipase (Lipozyme RM IM) (**Table 1**) at various temperatures: (a) 50, (b) 60, and (c) 80 °C in vacuo (20 hPa) or (d) 80 °C without vacuum. Our results show that the highest proportions of diacylglycerols (>70%) are formed by lipase-catalyzed transesterification at 50 and 60 °C (**Figure 1a**,**b**). In addition, the proportion of unesterified fatty acids is somewhat increased in experiments without vacuum (**Figure 1d**) as compared to those under vacuum at 50 and 60 °C (**Figure 1a**,**b**).

Figure 2 shows the time course of the formation of diacylglycerols via transesterification of 300 μmol of Mulgaprime 90 monoacylglycerols with 100 μmol of rapeseed oil triacylglyc-

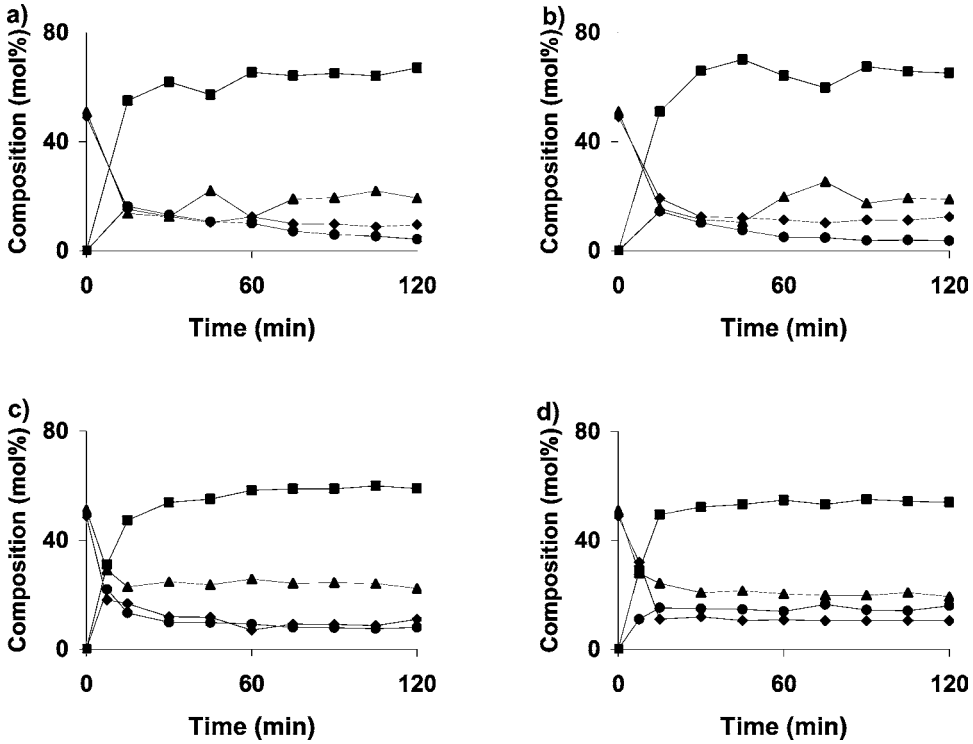

Figure 1. Time course of the formation of diacylglycerols (■) and fatty acids (●) via transesterification of 0.3 mmol (106 mg) of Monomuls 90-018 monoacylglycerols (◆) with 0.1 mmol (88 mg) of rapeseed oil triacylglycerols (▲) catalyzed by 25 mg (13 wt % enzyme based on weight of substrates) of immobilized *R. miehei* lipase (Lipozyme RM IM) at various temperatures, (**a**) 50, (**b**) 60, and (**c**) 80 °C, in vacuo (20 hPa) or (**d**) at 80 °C without vacuum.

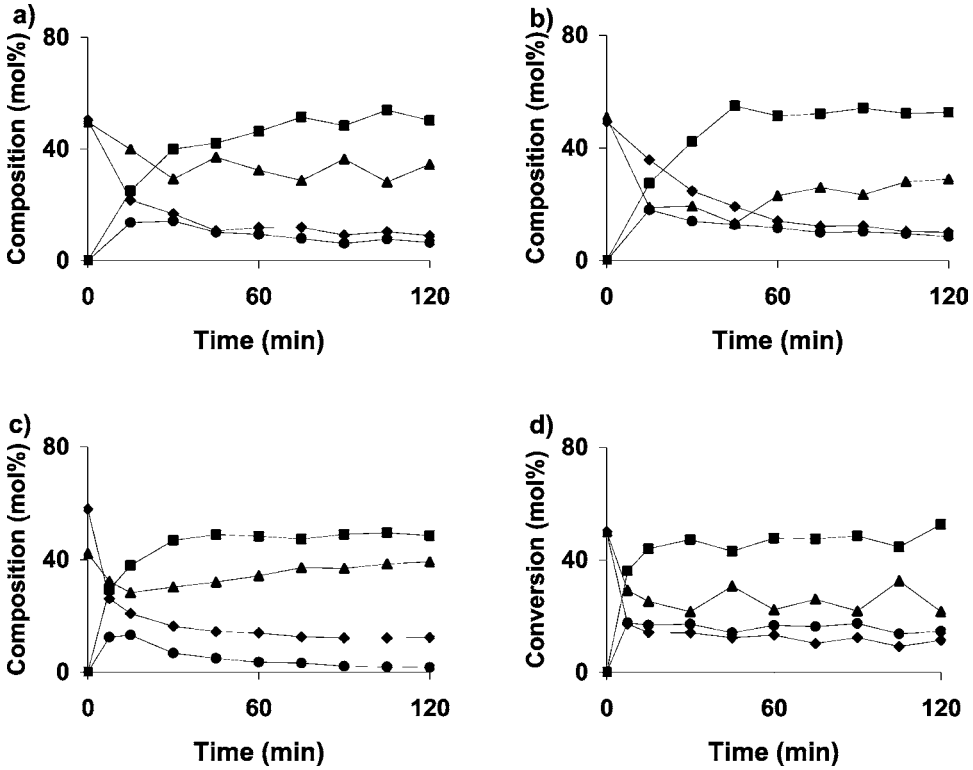

Figure 2. Time course of the formation of diacylglycerols (■) and fatty acids (●) via transesterification of 0.3 mmol (102 mg) of Mulgaprime 90 monoacylglycerols (◆) with 0.1 mmol (88 mg) of rapeseed oil triacylglycerols (▲) catalyzed by 25 mg (13 wt % enzyme based on weight of substrates) of immobilized *R. miehei* lipase (Lipozyme RM IM) at various temperatures, (**a**) 50, (**b**) 60, and (**c**) 80 °C, in vacuo (20 hPa) or (**d**) at 80 °C without vacuum.

erols, catalyzed by 25 mg of *R. miehei* lipase (Lipozyme RM IM) (**Table 1**) at various temperatures: (a) 50, (b) 60, and (c) 80 °C in vacuo (20 hPa) or (d) at 80 °C without vacuum. The lipase-catalyzed transesterification of Mulgaprime monoacylglycerols (**Figure 2**) shows results similar to those found for the experiment with Monomuls monoacylglycerols (**Figure 1**).

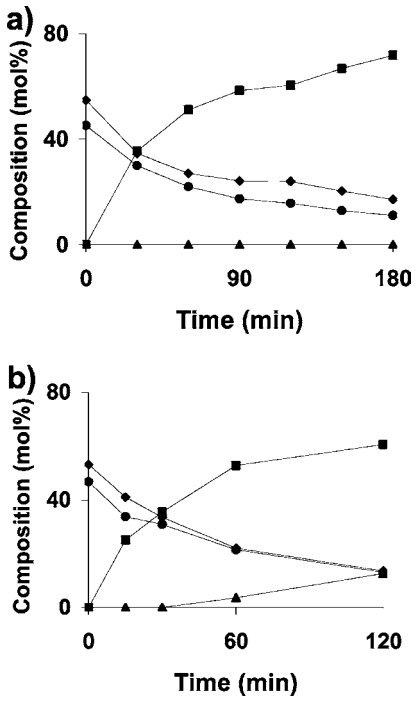

Figure 3. Time course of the formation of diacylglycerols (■) and triacylglycerols (▲) via esterification of 1 mmol (340 mg) of Nutrisoft 55 monoacylglycerols (◆) with 1 mmol (283 mg) of rapeseed oil fatty acids (●) catalyzed by 57 mg (9 wt % enzyme based on weight of substrates) of immobilized *R. miehei* lipase (Lipozyme RM IM) at various temperatures, (**a**) 60 and (**b**) 70 °C, in vacuo (20 hPa).

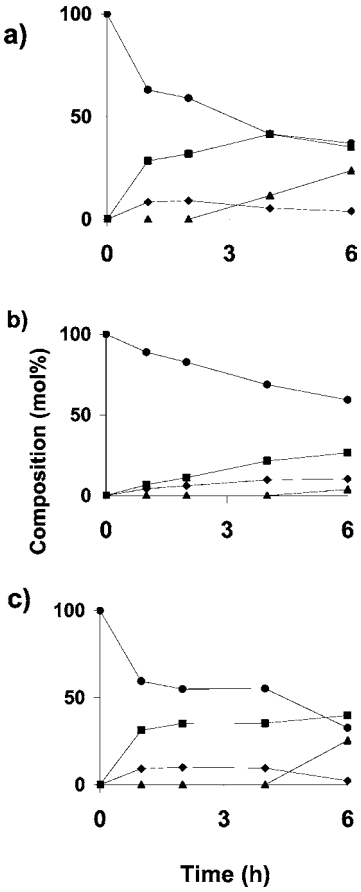

Figure 4. Time course of the formation of monoacylglycerols (◆), diacylglycerols (■), and triacylglycerols (▲) via esterification of 3 mmol (849 mg) of rapeseed oil fatty acids (●) with 1 mmol (92 mg) of glycerol (adsorbed to 180 mg of silica gel), catalyzed by various immobilized lipases, (**a**) *R. miehei* (Lipozyme RM IM), (**b**) *T. lanuginosus* (Lipozyme TL IM), and (**c**) *C. antarctica* (lipase B, Novozym 435), 84 mg each (9 wt % enzyme based on weight of substrates), in vacuo (20 hPa) at 60 °C.

However, the maximum proportions of diacylglycerols (~60%) formed in the reactions of Mulgaprime monoacylglycerols (**Figure 2**) are lower, as compared to Monomuls monoacylglycerols (**Figure 1**), which may be attributed to the relatively high melting point of Mulgaprime monoacylglycerols that hampers the ease of reaction.

Esterification of Monoacylglycerols with Rapeseed Oil Fatty Acids. Figure 3 shows the time course of the formation of diacylglycerols via esterification of 1 mmol of Nutrisoft 55 monoacylglycerols with 1 mmol of rapeseed oil fatty acids catalyzed by 57 mg of *R. miehei* lipase (Lipozyme RM IM) (**Table 1**) at various temperatures: (a) 60 and (b) 70 °C in vacuo (20 hPa). This experiment demonstrates that lipase-catalyzed esterification of monoacylglycerols with fatty acids at 60 °C yields similar proportions of diacylglycerols (>70%) (**Figure 3**) as observed for transesterification reactions of Monomuls monoacylglycerols (**Figure 1**). Moreover, the reaction temperature of 60 °C should be favored over higher temperatures because of very low formation of triacylglycerol byproducts (**Figure 3**).

Esterification of Glycerol with Rapeseed Oil Fatty Acids. Figure 4 shows the time course of the formation of monoacylglycerols, diacylglycerols, and triacylglycerols via esterification of 3 mmol of rapeseed oil fatty acids with 1 mmol of glycerol (adsorbed to silica gel), catalyzed by various immobilized lipases (**Table 1**): (a) *R. miehei* (Lipozyme RM IM), (b) *T. lanuginosus* (Lipozyme TL IM), and (c) *C. antarctica* (lipase B, Novozym 435), 84 mg each, in vacuo (20 hPa) at 60 °C. It is evident from these results that the formation of diacylglycerols is substantially lower in these experiments (**Figure 4**) as compared to esterification or transesterification of monoacylglycerols as described above (**Figures 1**−**3**). Best results (40−45% diacylglycerols) are obtained in reactions catalyzed by Lipozyme RM IM (**Figure 4a**) and Novozym 435 (**Figure 4c**), whereas only small proportions of diacylglycerols (25% at maximum) were observed in Lipozyme TL IM-catalyzed rections (**Figure 4b**). It should be noted, though, that the use of silica gel involves additional processing for its removal from the reaction products.

Glycerolysis of Rapeseed Oil Triacylglycerols. Figure 5 shows the time course of the formation of monoacylglycerols, diacylglycerols, and fatty acids via glycerolysis of 0.5 mmol of rapeseed oil triacylglycerols with 0.5 mmol of glycerol (adsorbed to silica gel), catalyzed by various immobilized lipases (**Table 1**): (a) *C. rugosa*, (b) *T. lanuginosus* (Lipozyme TL IM), and (c) *C. antarctica* (lipase B, Novozym 435), 42 mg each, in vacuo (20 hPa) at 60 °C. These experiments show that only the glycerolysis catalyzed by Lipozyme TL IM and Novozym 435 leads to moderate proportions (around 40 and 50%, respectively) of diacylglycerols, whereas *C. rugosa* lipase is least active in this reaction.

Addition of water to the lipase-catalyzed glycerolysis in the presence of silica gel (**Table 1**) led to low formation of diacylglycerols only (data not shown).

Repeated Use of Lipase in the Esterification of Monoacylglycerols with Rapeseed Oil Fatty Acids. Figure 6 shows the percent conversion of Monomuls 90-O18 monoacylglycerols to diacylglycerols and triacylglycerols in the esterification of monoacylglycerols (2 mmol) with rapeseed oil fatty acids (2 mmol), catalyzed by *R. miehei* lipase (Lipozyme RM IM, 60

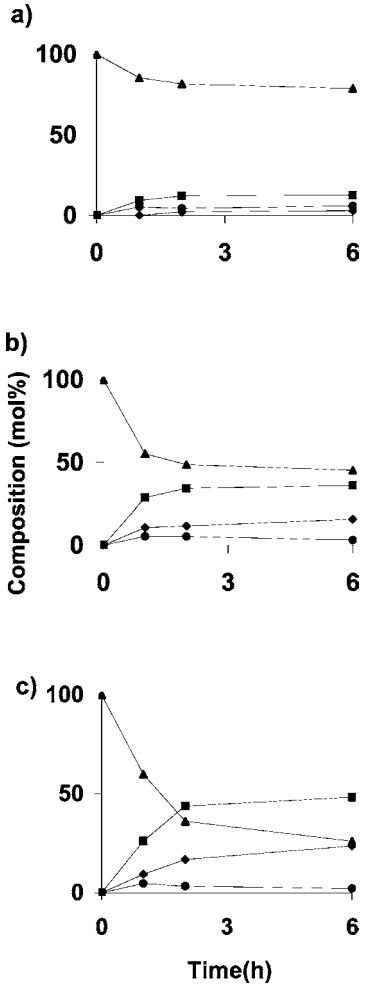

Figure 5. Time course of the formation of monoacylglycerols (◆), diacylglycerols (■), and fatty acids (●) via glycerolysis of 0.5 mmol (443 mg) of rapeseed oil triacylglycerols (▲) with 0.5 mmol (46 mg) of glycerol (adsorbed to 90 mg of silica gel), catalyzed by various immobilized lipases, (**a**) *C. rugosa*, (**b**) *T. lanuginosus* (Lipozyme TL IM), and (**c**) *C. antarctica* (lipase B, Novozym 435), 42 mg each (9 wt % enzyme based on weight of substrates), in vacuo (20 hPa) at 60 °C.

mg) after repeated use of the lipase catalyst as described under Materials and Methods. The reactions were carried out in vacuo (20 hPa) at 60 °C for 2 h, each. From the results given in **Figure 6** it is obvious that Lipozyme RM IM is stable as an enzyme catalyst at least over a period of 10 reaction cycles at 60 °C,

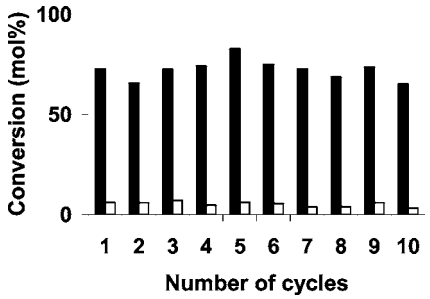

Figure 6. Percent conversion of Monomuls 90-O18 monoacylglycerols to diacylglycerols (■) and triacylglycerols (□) in the esterification of monoacylglycerols (2 mmol, 706 mg) with rapeseed oil fatty acids (2 mmol, 566 mg), catalyzed by *R. miehei* lipase (Lipozyme RM IM, 60 mg, 5 wt % enzyme based on weight of substrates) after repeated use of the lipase catalyst as described under Materials and Methods. The reactions were carried out in vacuo (20 hPa) at 60 °C for 2 h each.

leading to a 70% average conversion of monoacylglycerols to diacylglycerols together with small proportions of triacylglycerols.

Purification of Lipase-Catalyzed Reaction Products by Vacuum Distillation. Table 5 shows the composition of lipid fractions before and after vacuum distillation of various reaction mixtures from lipase-catalyzed transesterification or esterification of commercial monoacylglycerols or glycerol/silica gel with rapeseed oil triacylglycerols or fatty acids derived therefrom (**Table 1**). These results show that the mixtures of reaction products are partially purified by vacuum distillation. Under the conditions described, all reaction mixtures are almost completely freed from unesterified fatty acids. Under the conditions described, monoacylglycerols are partially removed, whereas triacylglycerols are not removed at all. Best results are obtained from the esterification of rapeseed oil fatty acids with Monomuls monoacylglycerols in the presence of Lipozyme RM IM, leading to a product that contains around 70% isomeric diacylglycerols, 19% triacylglycerols, and 11% monoacylglycerols (**Table 5**). Similarly, the distillation residue derived from the products of esterification of rapeseed oil fatty acids with glycerol contains around 66% isomeric diacylglycerols, 27% triacylglycerols, and 6% isomeric monoacylglycerols (**Table 5**).

In summary, our results show that interesterification of rapeseed oil triacylglycerols (containing 60% 18:1ω9, 19% 18:2ω6, and 8% 18:3ω3) with commercial preparations of monoacylglycerols, such as Monomuls 90-O18, Mulgaprime 90, and Nutrisoft 55, catalyzed by immobilized lipase from *R. miehei* (Lipozyme RM IM) in vacuo at 60 °C leads to extensive (from

Table 5. Composition of Lipid Fractions before and after Vacuum Distillation of Various Reaction Products from Lipase-Catalyzed Transesterification or Esterification of Commercial Monoacylglycerols or Glycerol/Silica Gel with Rapeseed Oil Triacylglycerols or Fatty Acids Derived Therefrom

starting materials	reaction mixture or distillation fraction	lipid fractions[a] (wt %)			
		MAG	DAG	FA	TAG
Mulgaprime (1 mmol, 342 mg) +	reaction mixture	9.0	33.8	1.8	55.3
rapeseed oil triacylglycerols	distillate	79.8	nd	20.2	nd
(1 mmol, 887 mg)[b]	distillation residue	1.9	31.5	0.3	66.2
Monomuls (1 mmol, 353 mg) +	reaction mixture	14.9	63.8	18.6	2.7
rapeseed oil triacylglycerols	distillate	22.0	nd	77.9	nd
(1 mmol, 887 mg)[b]	distillation residue	10.9	69.9	nd	19.2
glycerol (2 mmol, 184 mg)/silica gel (360 mg) +	reaction mixture	5.9	35.7	40.0	18.3
rapeseed oil fatty acids	distillate	6.3	nd	93.7	nd
(4 mmol, 1012 mg)[c]	distillation residue	6.0	65.7	1.8	26.5

[a] Abbreviations: DAG, diacylglycerols; FA, unesterified fatty acids; MAG, monoacylglycerols; TAG, triacylglycerols; nd, not detected. [b] Immobilized lipase used: 85 mg of Lipozyme RM IM (7 wt % enzyme based on weight of substrates) for 2 h at 60 °C in vacuo (20 hPa). [c] Immobilized lipase used: 112 mg of Novozym 435 (9 wt % enzyme based on weight of substrates) for 1 h at 60 °C in vacuo (20 hPa).

60% to a maximum of 80%) formation of diacylglycerols. Similarly, esterification of rapeseed oil fatty acids with Nutrisoft monoacylglycerols, catalyzed by Lipozyme RM in vacuo at 60 °C, also leads to remarkably high (60−70%) formation of diacylglycerols. Free fatty acids present in the products resulting from esterification and transesterification reactions as described above can be removed by short-path vacuum distillation leading to distillation residues (DAG oils) containing up to 70% diacylglycerols besides minor proportions of triacylglycerols (19−26%) and monoacylglycerols (6−11%).

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LITERATURE CITED

(1) Nagao, T.; Watanabe, H.; Goto, N.; Onizawa, K.; Taguchi, H.; Matsuo, N.; Yasukawa, T.; Tsushima, R.; Shimasaki, H.; Itakura, H. Dietary diacylglycerol suppresses accumulation of body fat compared to triaglycerol in men in a double-blind controlled trial. *J. Nutr.* **2000**, *130*, 792−797.

(2) Matsuo, N.; Tokimitsu, I. Metabolic characteristics of diacylglycerol. An edible oil that is less likely to become body fat. *Int. News Fats Oils Relat. Mater.* **2001**, *12*, 1098−1102.

(3) Murase, T.; Mizuno, T.; Omachi, T.; Onizawa, K.; Komine, Y.; Kondo, H.; Hase, T.; Tokimitsu, I. Dietary diacylglycerol suppresses high fat and high sucrose diet-induced body fat accumulation in C57BL/6J mice. *J. Lipid Res.* **2001**, *42*, 372−378.

(4) Murase, T.; Aoki, M.; Wakisaka, T.; Hase, T.; Tokimitsu, I. Anti-obesity effect of dietary diacylglycerol in C57BL/6J mice: dietary diacylglycerol stimulates intestinal lipid metabolism. *J. Lipid Res.* **2002**, *43*, 1312−1319.

(5) Maki, K. C.; Davidson, M. H.; Tsushima, R.; Matsuo, N.; Tokimitsu, I.; Umporowicz, D. M.; Dicklin, M. R.; Foster, G. S.; Ingram, K. A.; Anderson, B. D.; Frost, S. D.; Bell, M. Consumption of diacylglycerol oil as part of a reduced-energy diet enhances loss of body weight and fat in comparison with consumption of a triacylglycerol control oil. *Am. J. Clin. Nutr.* **2002**, *76*, 1230−1236.

(6) Flickinger, B. D.; Matsuo, N. Nutritional characteristics of DAG oil. *Lipids* **2003**, *38*, 129−132.

(7) Taguchi, H.; Watanabe, H.; Onizawa, K.; Nagao, T.; Gotoh, N.; Yasukawa, T.; Tsushima, R.; Shimasaki, H.; Itakura, H. Double-blind controlled study on the effects of dietary diacylglycerol on postprandial serum and chylomicron triacylglycerol responses in healthy humans. *J. Am. Coll. Nutr.* **2000**, *19*, 789−796.

(8) Yamamoto, K.; Asakawa, H.; Tokunaga, K.; Watanabe, H.; Matsuo, N.; Tokimitsu, I.; Yagi, N. Long-term ingestion of dietary diacylglycerol lowers serum triacylglycerol in type II diabetic patients with hypertriglyceridemia. *J. Nutr.* **2001**, *131*, 3204−3207.

(9) Tada, N.; Yoshida, H. Diacylglycerol on lipid metabolism. *Curr. Opin. Lipidol.* **2003**, *14*, 29−33.

(10) Taguchi, H.; Omachi, T.; Nagao, T.; Matsuo, N.; Tokimitsu, I.; Itakura, H. Dietary diacylglycerol suppresses high fat diet-induced hepatic fat accumulation and microsomal triacylglycerol transfer protein activity in rats. *J. Nutr. Biochem.* **2002**, *13*, 678−683.

(11) Sakaguchi, H. Marketing a healthy oil. *Oils Fats Int.* **2001**, 18−19.

(12) Watkins, C. Time for an oil change. *Int. News Fats Oils Relat. Mater.* **2003**, *14*, 70.

(13) Mukherjee, K. D. Lipase-catalyzed reactions for modification of fats and other lipids. *Biocatalysis* **1990**, *3*, 277−293.

(14) Bornscheuer, U. T. Lipase-catalyzed syntheses of monoacylglycerols. *Enzyme Microb. Technol.* **1995**, *17*, 578−586.

(15) Rendon, X.; López-Munguía, A. Solvent engineering applied to lipase-catalyzed glycerolysis of triolein. *J. Am. Oil Chem. Soc.* **2001**, *78*, 1061−1066.

(16) Liao, F.-F.; Tsai, W.-C.; Chang, S.-W.; Shieh, C.-J. Application of solvent engineering to optimize lipase-catalyzed 1,3-diacylglycerols by mixture response surface methodology. *Biotechnol. Lett.* **2003**, *25*, 1857−1861.

(17) Rosu, R.; Yasui, M.; Iwasaki, Y.; Yamane, T. Enzymatic synthesis of symmetrical 1,3-diacylglycerols by direct esterification of glycerol in solvent-free system. *J. Am. Oil Chem. Soc.* **1999**, *76*, 839−843.

(18) Watanabe, T.; Shimizu, M.; Sugiura, M.; Sato, M.; Kohori, J.; Yamada, N.; Nakanishi, K. Optimization of reaction conditions for the production of DAG using immobilized 1,3-regiospecific lipase Lipozyme RM IM. *J. Am. Oil Chem. Soc.* **2003**, *80*, 1201−1207.

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