Solvent-Free Enzymatic Glycerolysis of Marine Oils

Bjørnar Myrnes*, Harald Barstad, Ragnar L. Olsen and Edel O. Elvevoll

Norwegian Institute of Fisheries and Aquaculture, Breivika, N-9002 Tromsø, Norway

ABSTRACT: Marine triglyceride oils (cod liver oil and oils from blubber of harp seal and minke whale) were reacted with glycerol using lipase as a catalyst at low temperature. A solvent-free batch system with magnetic stirring was used. Solidification of the reaction mixture occurred, and a mixture of mono-, di-, and triglycerides was obtained in all cases. The recovered glyceride mixtures were solid at room temperature. The yield of monoglyceride (MG) and the fatty acid profile of the MG fractions were dependent on oil and the type of lipase used as a catalyst. Of the commercially-available lipases investigated, lipase AK from *Pseudomonas* sp. synthesized the highest yield of MG (42–53%) at 5°C. These MG fractions were low in saturated fatty acids (4–11%) and high in long-chain monounsaturated fatty acids (52–69%). The concentration of n-3 polyunsaturated fatty acids was 12–20%. *JAOCS 72*, 1339–1344 (1995).

KEY WORDS: Glycerolysis, lipase, marine oils, monoglyceride, solidification.

Monoglycerides (MG) are widely used as emulsifiers in the food and pharmaceutical industries (1,2). A widely used method of commercial MG synthesis involves heating a mixture of triglyceride (TG)-rich oil and glycerol to approximately 220°C using an alkaline catalyst (1). This method suffers from several drawbacks, including formation of dark-colored by-products with a strong off-flavor which have to be removed using molecular distillation. To overcome these disadvantages, synthesis of MG using lipase (EC 3.1.3.3) as catalyst has been investigated. Earlier attempts to synthesize MG using lipase as a catalyst resulted in low yield (3,4) or required the use of organic solvents (5). Recently, a system for the solvent-free glycerolysis of fat and oils giving a high yield of MG has been developed by McNeill and co-workers (6–8). They showed that a high MG yield is only obtained when the reaction is carried out below a critical temperature (7).

In recent years the health aspects of marine fat have been studied extensively (9–11). The beneficial effects have been attributed to the long-chain n-3 polyunsaturated fatty acids (PUFA), characteristic of marine fat. The n-3 PUFA are highly labile, and procedures that involve extremes of pH or high temperature are usually detrimental due to destruction of their natural all-*cis* ω-3 framework by oxidation, *cis–trans* isomerization, or double-bond migration. Recently, monoglycerides significantly enriched in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by lipase-catalyzed alcoholysis of cod liver oil (12) and esterification of glycerol and n-3 PUFA in organic solvent (13,14) have been reported. The substrates for enzymatic glycerolysis are TG and glycerol, in contrast to enzymatic ester synthesis, which require expensive n-3 PUFA as substrate. The work reported here describes the application of solvent-free enzymatic glycerolysis on marine oils.

EXPERIMENTAL PROCEDURES

Materials. Commercially-available lipases (E.C. 3.1.1.3) were used. *Pseudomonas* sp. lipase AK (lot: LAKQ08506), *Rhizopus niveus* lipase N (lot: LNQ11510), and *R. delemar* lipase D (lot: 20161TM20) were donated by Amano Enzyme Europe Ltd. (Milton Keynes, England). *Chromobacterium viscosum* lipase CV (lot: L050P) was donated by Biocatalysts Ltd. (Mid Glamorgan, England). Glycerol (p.a. grade) was purchased from BDH Inc. (Toronto, Canada). Cod liver oil and oils from blubber of harp seal and minke whale were prepared by low-temperature (<40°C) extraction. The whale blubber was obtained from animals hunted for research purposes in Norway in 1992, which was approved by the International Whaling Commission.

Glycerolysis. A mixture of glycerol, water, lipase powder, and oil was prepared as described by McNeill and co-workers (6). Unless otherwise stated, water was dissolved in 5 g glycerol to give a final moisture content of 3.6%. Lipase powder (200 mg) was suspended in the glycerol/water solution, and 23 g oil was added. The enzymes were used without any further purification or modification. The reactions were carried out in covered, flat-bottomed reaction beakers (4 × 10 cm) at 5°C with magnetic stirring (300 rpm).

Analysis. During the course of the glycerolysis reaction, samples of approximately 100 μL were withdrawn from the reaction vessel and subjected to chloroform extraction. The extract was analyzed for TG, 1,3-diglyceride (1,3-DG), 1,2-diglyceride (1,2-DG), MG, and free fatty acid (FFA) by thin-layer chromatography (TLC)/flame-ionization detection (Iatroscan TH-10; Iatron Laboratories, Tokyo, Japan).

Chloroform extract (0.3 μL) was applied to a Chromarod S III (Iatron) quartz rod followed by development in hexane/chloroform/formic acid (35:35:0.14, vol/vol/vol). The rods were

*To whom correspondence should be addressed at Fiskeriforskning, P.O. Box 2511, N-9002 Tromsø, Norway.

dried and scanned using Iatroscan TH-10. Peak areas were calculated using a Spectra-Physics SP 4270 integrator (Spectra-Physics, Autolab Division, San Jose, CA). Results are expressed as % peak areas.

Semi-preparative separation and recovery of MG fractions was carried out by TLC. Chloroform extract (200 μL) of the reaction mixture at equilibrium was applied as a band on a 5 × 20 cm TLC plate Kiselgel 60, 0.25-mm layer containing fluorescent indicator (G. Merck, Darmstadt, Germany). After development in chloroform/acetone (96:4, vol/vol) and drying at room temperature, the bands were visualized under ultraviolet light, scraped off, and extracted with chloroform.

The fatty acid composition of the oils and MG fractions was determined by capillary gas–liquid chromatography (GLC) using a Fisons Carlo Erba 8340 gas chromatograph equipped with an AS 800 auto injector (Carlo Erba, Italy). The oils and the recovered MG fractions were converted to glycerol and fatty acid methyl esters (FAME) by reacting the samples with an excess of 1 N alkaline methanol. After neutralizing the sample with hydrochloric acid, the methyl esters were extracted into heptane. Separation was carried out on a 50 m × 0.25 mm CP-sil 88 (FAME) capillary column with 0.2 μM film thickness (Chrompack, Middelburg, Holland) after splitless injection of 1 μL sample at a column temperature of 80°C. The carrier gas was hydrogen, with an inlet pressure of 110 kPa. The operating conditions were as follows: the initial temperature of 80°C was held for 1 min, then the temperature was raised by 20°C/min to 160°C, followed by a gradient of 2°C/min to 177°C and 20°C/min to 230°C, and was kept there for 15 min. The injector and the flame-ionization detector temperatures were 250 and 270°C, respectively. Integration was carried out using MAXIMA 820 (Millipore, Milford, MA).

Melting points were determined by the close capillary method (15) using a Büchi 535 apparatus (Büchi Laboratoriums-Technic AG, Flawil, Switzerland).

RESULTS

Glycerolysis of marine oils using commercial lipases. The glyceride composition of the reaction mixture after glycerolysis of cod liver oil (CLO), seal oil (SO), and whale oil (WO) at 5°C using two, 1,3-specific lipases (from *R. niveus* and *R. delemar*), and two nonspecific lipase (from *Pseudomonas* sp. and *C. viscosum*) as a catalyst is shown in Table 1. The temperature of the reaction mixtures prior to incubation was approximately 10°C. The incubation temperature was close to (for CLO) or below (for SO and WO) the melting point of these oils. After two to four days, depending on oil and lipase, the reaction mixture became solid, and further stirring was impossible. The rate of glycerolysis was slow and a mixture of TG, DG, and MG was obtained. Equilibrium was obtained after one to four weeks of incubation, depending on oil and lipase. The FFA level in the reaction mixtures at equilibrium was low (1.8–3.3%) using lipase AK, lipase N, and lipase D as a catalyst. The concentration of FFA was higher (5.8–8.3%) using *C. viscosum* lipase. Lipase AK produced the highest yield of MG in low-temperature glycerolysis of the marine oils. The major DG detected in the reaction mixture using the 1,3-specific lipases was 1,2-DG. The 1,3-DG/1,2-DG level was low (0.3–0.4) using the nonspecific lipase AK as a catalyst in contrast to the CV lipase (1.2–2.1). The reaction mixtures remained solid at room temperature. The melting point of the recovered glyceride mixtures after glycerolysis was between 35 and 50°C.

Time course of glycerolysis. The detailed change in composition of glycerides in lipase N- and AK-catalyzed glycerolysis of CLO is shown in Figures 1 and 2, respectively. The

TABLE 1
Equilibrium Composition of the Reaction Mixture After Glycerolysis of Cod Liver Oil, Seal Oil, and Whale Oil at 5°C Using Lipases as Catalyst

	Composition[a] (%)					
Lipase preparation	TG	1,3-DG	1,2-DG	MG	FFA	Time[b] (days)
Cod liver oil						
Rhizopus niveus	44.3	4.3	28.8	20.0	2.6	11
Chromobacterium viscosum	59.7	6.4	13.4	14.5	5.9	20
R. delemar	45.0	5.7	29.3	16.9	3.1	11
Pseudomonas sp.	22.8	20.8	8.2	45.9	2.3	15
Seal oil						
R. niveus	52.4	5.6	23.5	13.9	2.8	30
C. viscosum	52.5	10.5	13.9	17.3	5.8	21
R. delemar	49.2	8.2	25.1	14.6	2.9	11
Pseudomonas sp.	23.3	24.1	8.7	42.1	1.8	15
Whale oil						
R. niveus	43.5	5.1	27.5	20.6	3.3	21
C. viscosum	47.2	11.1	13.4	20.0	8.3	21
R. delemar	45.4	6.4	25.0	20.7	2.5	8
Pseudomonas sp.	18.9	18.3	6.2	53.3	3.3	21

[a]TG, triglyceride, DG, diglyceride; MG, monoglyceride; FFA, free fatty acids.

[b]Time is the minimum incubation time to reach equilibrium composition.

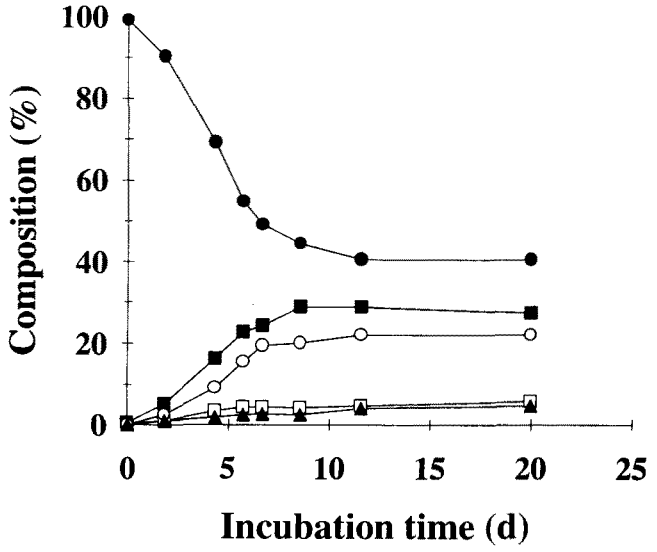

FIG. 1. Kinetics of lipase N, catalyzed glycerolysis of cod liver oil at 5°C: triglyceride (●), 1,3-diglyceride (□), 1,2-diglyceride (■), monoglyceride (○), free fatty acid (▲).

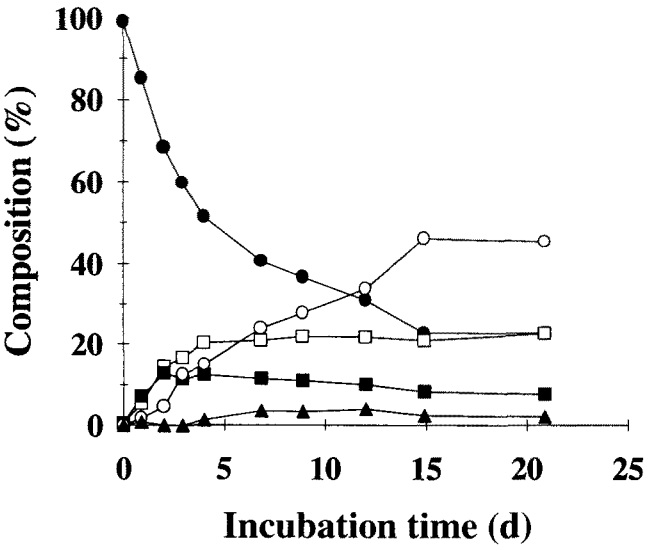

FIG. 2. Kinetics of lipase AK-catalyzed glycerolysis of cod liver oil at 5°C: triglyceride (●), 1,3-diglyceride (□), 1,2-diglyceride (■), monoglyceride (○), free fatty acid (▲).

reaction rate was low with both enzymes, but the initial rate of TG turnover and MG production was higher using lipase AK compared to using lipase N. Using lipase N as a catalyst (Fig. 1), the increase in 1,2-DG was accompanied by an accumulation of MG. The concentration of 1,3-DG remained low throughout the reaction. Equilibrium in the reaction mixture was reached after eleven days of incubation. 1,2-DG and 1,3-DG accumulated at the same rate as the first 48 h of incubation using lipase AK (Fig. 2). 1,3-DG increased in concentration up to four days of incubation and remained constant throughout the reaction. A slow decrease in 1,2-DG was observed. MG was accumulated in the reaction mixture at an al-

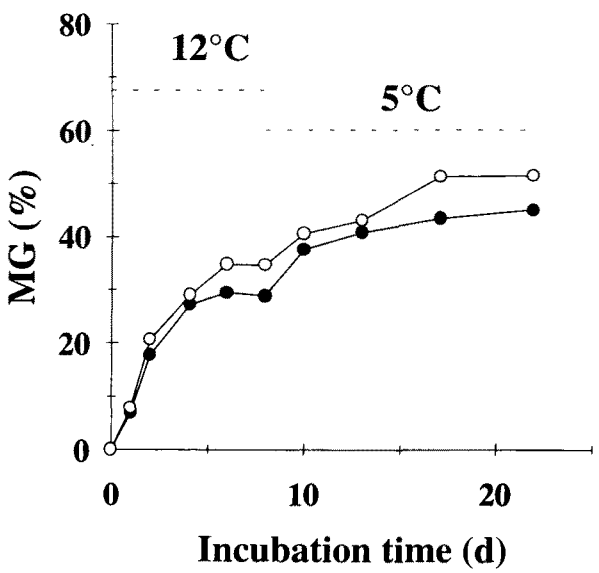

FIG. 3. The effect of temperature on monoglyceride (MG) production during lipase AK-catalyzed glycerolysis of cod liver oil (●) and whale oil (○) at 12°C, followed by incubation at 5°C.

most constant rate up to a final concentration of 45% at equilibrium. Almost identical reaction patterns were observed in whale oil and seal oil glycerolysis at 5°C.

Effect of temperature on MG production. In initial experiments, we observed very low yield of MG (~5%) when glycerolysis of CLO was performed at room temperature. Figure 3 shows the time course for the production of MG during glycerolysis of CLO and WO using lipase AK as a catalyst at 12°C, followed by 5°C incubation. Equilibrium was reached in the reaction mixtures after six days of incubation at 12°C. Reducing the incubation temperature resulted in complete solidification of the reaction mixtures, a new equilibrium composition, and a yield of MG from CLO and WO of 45 and 52%, respectively.

Effect of molar ratio of glycerol to CLO on glycerolysis. Figure 4 shows the equilibrium composition of lipase AK-catalyzed glycerolysis of CLO at different glycerol concentrations. As the glycerol concentration was increased to a mol ratio of glycerol/fat of 2:1, the yield of MG and 1,3-DG progressively increased. The level of 1,2-DG and FFA at equilibrium was independent of the glycerol concentration in the reaction mixture.

Fatty acid composition of MG fractions. Tables 2, 3, and 4 show the fatty acid composition of the marine oils in the original state and in the MG fractions of the reaction mixtures after separation by TLC. The FFA profile of the MG fractions varied both with oil and lipase used as a catalyst. Both the 1,3-specific and nonspecific lipases showed different affinity for selected fatty acids in TG of the oils. MG fractions with relatively high concentrations of n-3 PUFA and monoenoic fatty acids and low concentration of saturated fatty acids were obtained following glycerolysis of the marine oils using lipase AK. The MG fraction with the lowest concentration of satu-

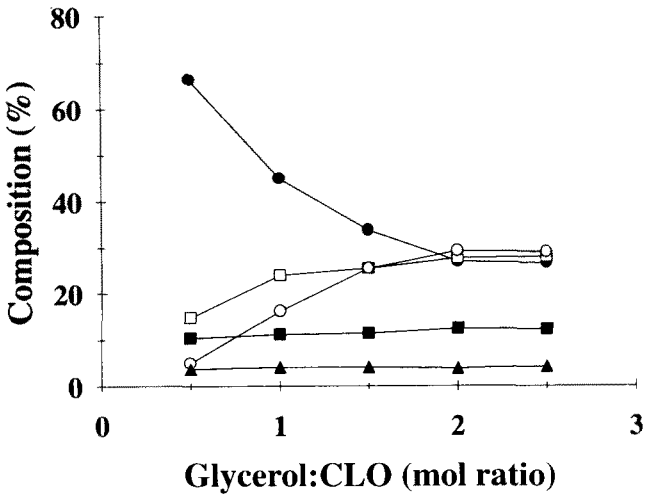

FIG. 4. Effect of glycerol content on the extent of glycerolysis. Reaction mixtures contained 2.3 g cod liver oil (CLO), 20 mg lipase AK, and various levels of glycerol (3.6% water), and were incubated for 180 h at 12°C. Triglyceride (●), 1,3-diglyceride (□), 1,2-diglyceride (■), monoglyceride (○), free fatty acid (▲).

TABLE 3
Fatty Acid Composition of Seal Oil and MG Fractions Obtained After Glycerolysis at 5°C with Lipases from *Rhizopus niveus* (N), *R. delemar* (D), *Chromobacterium viscosum* (CV), and *Pseudomonas* sp. (AK)[a]

	Seal oil	MG fractions			
Fatty acid		N	D	CV	AK
14:0	7.0	2.6	1.0	2.9	1.3
16:0	9.0	20.4	13.3	19.5	8.3
18:0	1.0	4.9	4.3	2.6	2.0
Σ saturated	17.0	27.9	18.6	25.0	11.6
16:1	13.0	4.9	5.8	7.2	6.3
18:1	17.5	16.5	19.4	19.5	20.7
20:1	12.8	13.6	21.9	13.1	16.1
22:1	5.6	5.8	7.8	4.8	8.4
Σ monoenes	48.9	40.8	54.9	44.6	51.5
18:3n-3	1.1	1.1	0.3	0.5	0.9
20:5n-3	7.5	5.0	7.5	5.9	7.0
22:5n-3	3.2	2.5	4.3	3.0	3.5
22:6n-3	9.5	3.5	6.2	10.1	9.4
Σ n-3 PUFA	21.8	12.1	18.3	19.5	20.8

[a]Abbreviations as in Tables 1 and 2.

TABLE 2
Fatty Acid Composition (%) of Cod Liver Oil (CLO) and MG Fractions Obtained After Glycerolysis at 5°C with Lipases from *Rhizopus niveus* (N), *R. delemar* (D), *Chromobacterium viscosum* (CV), and *Pseudomonas* sp. (AK)[a]

	CLO	MG fractions			
Fatty acid		N	D	CV	AK
14:0	4.6	2.8	4.0	6.7	0.5
16:0	10.4	16.5	23.2	34.4	6.6
18:0	2.6	7.8	11.1	6.3	3.7
Σ saturated	17.6	27.1	38.3	47.4	10.8
16:1	10.4	6.0	5.3	5.3	3.7
18:1	21.3	20.3	14.0	14.1	18.6
20:1	12.0	16.8	10.7	9.7	20.3
22:1	5.0	7.6	5.0	4.0	11.2
Σ monoenes	48.7	50.7	35.0	33.1	53.8
18:3n-3	0.8	0.7	1.3	0.6	0.7
20:5n-3	8.8	4.9	5.3	4.1	8.0
22:5n-3	1.0	0.7	0.7	0.5	1.0
22:6n-3	11.9	7.2	6.9	5.7	9.8
Σ n-3 PUFA	22.5	13.5	14.2	10.9	19.5

[a]Abbreviation as in Table 1. *PUFA, polyunsaturated fatty acid.*

TABLE 4
Fatty Acid Composition (%) of Whale Oil and MG Fractions Obtained After Glycerolysis at 5°C with Lipases from *Rhizopus niveus* (N), *R. delemar* (D), *Chromobacterium viscosum* (CV), and *Pseudomonas* sp. (AK)[a]

	Whale oil	MG fractions			
Fatty acid		N	D	CV	AK
14:0	6.7	4.2	2.0	5.2	0.3
16:0	10.2	11.0	17.6	8.3	2.4
18:0	2.4	3.7	8.5	1.5	1.6
Σ saturated	19.3	18.9	28.1	15.0	4.3
16:1	9.7	9.6	3.4	12.3	2.8
18:1	21.4	20.2	12.6	23.1	15.1
20:1	15.5	17.1	18.4	14.0	26.3
22:1	9.5	9.4	15.4	5.8	24.6
Σ monoenes	56.1	56.3	49.8	55.2	68.8
18:3n-3	1.3	0.6	1.3	0.4	0.9
20:5n-3	3.8	4.3	2.9	6.4	3.8
22:5n-3	1.6	2.0	1.7	3.0	2.3
22:6n-3	5.1	6.2	1.9	7.5	5.4
Σ n-3 PUFA	11.8	13.1	7.8	17.3	12.1

[a]Abbreviations as in Tables 1and 2.

rated fatty acids (4.3%) and highest concentration of unsaturated fatty acids was obtained from the whale oil reaction mixture (Table 4). The CV lipase synthesized an MG fraction in whale oil glycerolysis with increased n-3 PUFA and less saturated fatty acids compared to the original oil. MG fractions containing ~20% n-3 PUFA were obtained from seal oil using lipase D and CV as a catalyst (Table 3). The MG fractions obtained from the CLO reaction mixtures using lipase N, D, and CV as a catalyst contained higher amounts of saturated fatty acids and less n-3 PUFA than the original oil (Table 2). The MG fraction with the lowest concentration of n-3 PUFA was obtained following glycerolysis of whale oil using lipase D (Table 4).

DISCUSSION

In this paper it has been shown that MG can be obtained by solvent-free glycerolysis of marine oils using lipase as a catalyst. At low reaction temperature, a yield of approximately 40–50% MG can be synthesized, which was higher than reported in lipase-catalyzed alcoholysis (12) and selective hydrolysis (16) of CLO. The yield of MG at equilibrium was de-

pendent on lipase used as catalyst; the highest yields were obtained from CLO (46%), SO (42%), and WO (53%) using a lipase from *Pseudomonas* sp. Lipases from *P. fluorescens* and *C. viscosum* are reported to be the most efficient catalysts in enzyme-catalyzed glycerolysis of various fats and plant oils (6,7). The *C. viscosum* lipase was, in our study, a considerably less efficient catalyst than the *Pseudomonas* sp. lipase in glycerolysis of the marine oils. As expected, the 1,3-specific lipases produced relatively more 1,2-DG as compared with 1,3-DG. A difference in 1,2-DG and 1,3-DG levels at equilibrium was observed regarding the nonspecific lipases. The *C. viscosum* lipase produced nearly twice the amount of 1,3-DG as the AK lipase did and behaved more like a 1,3-specific lipase in glycerolysis of CLO at low temperature.

A longer incubation time was needed to obtain equilibrium in the marine oil reaction mixtures than reported for *P. fluorescens* lipase-catalyzed glycerolysis of plant oils at 5°C (7). The kinetics of the *Pseudomonas* sp. lipase AK-catalyzed glycerolysis of CLO at 5°C was also different than reported for the *P. fluorescens* lipase-catalyzed glycerolysis of tallow (6) and palm oil (7) at 40°C. Both MG and DG were produced during the early stage of the CLO glycerolysis, as in the palm oil and tallow glycerolysis. But further synthesis of MG was not accompanied by a subsequent decrease in DG concentration, as observed for tallow and palm oil. Only a slow decrease in 1,2-DG concentration was observed in the reaction mixture during incubation. In the lipase N-catalyzed glycerolysis of CLO, no decrease in DG was observed during incubation. This indicates that MG is mainly produced by the reaction of 1 mol of glycerol and 1 mol of TG in CLO glycerolysis at low temperature.

The lipases used as a catalyst in glycerolysis of the marine oils showed different affinity for selected fatty acids in the TG of the oils. The FFA profile of the MG fractions shows that lipase AK from *Pseudomonas* sp. catalyzes transfer of saturated fatty acids to glycerol less frequently than the other lipases used in this study. This lipase also shows a considerably lower affinity for these fatty acids in TG of whale oil compared to SO and CLO. The concentration of n-3 PUFA was not increased in the MG fractions compared to the original oils using this enzyme. In a recent paper by Li and Ward (12), high concentrations of EPA (20:5) and DHA (22:6) in MG were obtained using lipase CES from *Pseudomonas* sp. as a catalyst in alcoholysis of CLO at low temperature. A marked difference in affinity for selected fatty acids was also observed for the *C. viscosum* lipase. The most striking difference in fatty acid affinity was observed regarding palmitic acid (16:0) in TG of CLO and WO. Although the concentration of this fatty acid is the same in TG of the oils, a fourfold higher concentration of palmitic acid was detected in the MG fraction obtained from the CLO glycerolysis compared to the MG fraction from WO glycerolysis. The differences observed regarding fatty acid affinity for the lipases used in marine oil glycerolysis may reflect different positions of selected fatty acids on TG of the marine oils (17,18).

Reducing the incubation temperature in lipase AK-catalyzed glycerolysis of CLO and WO resulted in new equilibrium compositions and higher yields of MG. This indicates a critical temperature for MG synthesis close to the melting point of the oils using lipase AK as a catalyst, as reported for other fats and oils using *P. fluorescens* lipase as a catalyst (6,7). In enzymatic glycerolysis of animal fats and plant oils below a critical temperature, high yield of MG and solidification of the reaction mixture were attributed to the preferential distribution of saturated fatty acids in the MG fraction (19,20). In our study on the glycerolysis of marine oils with lipase AK, high yield of MG and solidification also occurred. In this case, however, the MG were very low in saturated fatty acids and high in unsaturated fatty acids. Solidification of the reaction mixtures also occurred when lipases N, CV, and D were used as a catalyst in low-temperature glycerolysis of the marine oils. The fact that the recovered glyceride mixtures were solid at room temperature indicates that mechanisms other than crystallization of MG, high in saturated fatty acids, are involved in solidification of the marine oil glycerolysis mixtures. The enzymatic hardening of the marine oil glyceride mixtures observed in this work has to be investigated further.

Efficient, solvent-free glycerolysis of marine oils at low temperature (5–10°C) eliminates the possibility of double-bond oxidation which might easily occur at the reaction temperature of chemical glycerolysis. Enzymatic glycerolysis could therefore be an approach to produce favorable MG low in saturated and high in unsaturated fatty acids from marine oils.

ACKNOWLEDGMENT

This work was supported by a research grant from the Norwegian Research Council.

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[Received March 6, 1995; accepted July 10, 1995]