The Preparation of Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase-Catalyzed Transesterification of Fish Oil with Ethanol

Gudmundur G. Haraldsson[a,*], Björn Kristinsson[a], Ragnheidur Sigurdardottir[a], Gudmundur G. Gudmundsson[a], and Harald Breivik[b]

[a]Science Institute, University of Iceland, Reykjavik, Iceland, and [b]Norsk Hydro ASA, Research Centre Porsgrunn, Porsgrunn, Norway

ABSTRACT: The objective of this study was to investigate the use of lipases as catalysts for producing concentrates of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil as an alternative to conventional chemical procedures. Transesterification of fish oil with ethanol was conducted under anhydrous solvent-free conditions with a stoichiometric amount of ethanol. Among the 17 lipases tested, the results showed that *Pseudomonas* lipases had the highest activity toward the saturated and monounsaturated fatty acids in the fish oil, much lower activity toward EPA and DHA and, at the same time, good tolerance toward the anhydrous alcoholic conditions. With 10 wt% of lipase, based on weight of the fish oil triacylglycerol substrate (15% EPA and 9% DHA initial content), a 50% conversion into ethyl esters was obtained in 24 h at 20°C, in which time the bulk of the saturated and monounsaturated fatty acids reacted, leaving the long-chain n-3 polyunsaturated fatty acids unreacted in the residual mixture as mono-, di-, and triacylglycerols. This mixture comprised approximately 50% EPA + DHA. Total recovery of DHA and EPA was high, over 80% for DHA and more than 90% for EPA. The observed fatty acid selectivity, favoring DHA as a substrate, was most unusual because most lipases favor EPA.

JAOCS 74, 1419–1424 (1997).

KEY WORDS: DHA, EPA, ethanolysis, n-3 fatty acids, fish oil, lipase, *Pseudomonas fluorescens* lipase (PFL), *Pseudomonas* species lipase (PSL), transesterification.

The beneficial health effects of the long-chain polyunsaturated n-3 fatty acids that are characteristic of marine lipids, especially *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), are now well established (1). Consequently, there is a growing demand for concentrates of these fatty acids. The chief objective of the research described in this report was to investigate the possibility of using lipases as catalysts for producing concentrates of EPA and DHA from fish oil raw material as an alternative to existing industrial processes. Lipases offer the following advantages over currently used chemical methods (2,3). First, lipase catalytic efficiency is high, so a relatively low amount of enzyme is required, especially when immobilized, which can be reused. Second, their wide range in fatty acid selectivity is well known and is crucial for the application intended. The mildness that lipases and other enzymes offer in terms of temperature, pH, and pressure is also important in processes that involve the highly labile n-3 type polyunsaturated fatty acids. Their all-*cis* n-3 structure is prone to partial destruction by oxidation, *cis-trans* isomerization, or double-bond migration, and they are also susceptible to polymerization because of the pH and high temperature required in conventional chemical processes. Reduced energy cost also may be an important advantage. Finally, the fact that lipase-promoted esterifications can be conducted under solvent-free conditions may result in a considerable reduction of the bulkiness of the process.

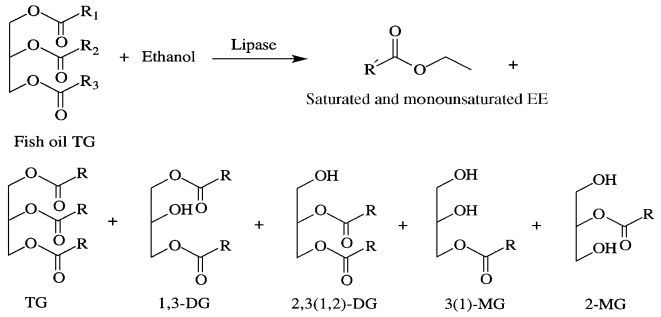
Numerous reports in the literature involve biotransformations of n-3 polyunsaturated fatty acids from marine oil by lipases. Many of the commercially available lipases discriminate against n-3 polyunsaturated fatty acids (3). This has enabled their use to concentrate both EPA and DHA by hydrolysis of fish oils (4,5). Virtually all lipases that display any significant activity toward n-3 fatty acids, however, also discriminate between EPA and DHA in favor of the former. This has allowed a number of groups to prepare concentrates of EPA and DHA by discriminating between these two during lipase hydrolysis of fish oil triacylglycerols (TG), usually with an emphasis on DHA enrichment for infant formula development (6–10).

Lipases have been employed for the enrichment of fish oil TG with n-3 concentrates by various transesterification reactions (11,12). The preparation of homogeneous TG that contain both EPA and DHA also has been described by direct esterification of glycerol with pure EPA and DHA as free acids as well as by interesterification of tributyrin with ethyl esters (EE) of pure EPA and DHA (13). Recently, Shimada and coworkers (14) reported a highly efficient concentration of DHA by direct esterification of the free fatty acids (FFA) from tuna oil with various alcohols by means of lipases. There are other related reports on the direct esterification of polyunsaturated FFA from fish oil with simple alcohols (15–18).

*To whom correspondence should be addressed at Science Institute, University of Iceland, Dunhaga 3, 107 Reykjavik, Iceland.
E-mail: gghar@raunvis.hi.is.

We were interested in concentrating both EPA and DHA in fish oil. Accordingly, a lipase was needed that would act preferentially toward the saturated and monounsaturated fatty acids in fish oil, leaving the polyunsaturated fatty acids behind, including both EPA and DHA. In addition, minimum discrimination by the lipase between EPA and DHA was required, as well as a good tolerance of the lipase toward fish oil TG and the reaction conditions.

The selected reaction was the transesterification of the initial fish oil triglycerides with ethanol. This ethanolysis reaction was conducted under water-deficient solvent-free conditions, which reduced the bulkiness of the process to a considerable extent. Most of the saturated and monounsaturated fatty acids of the fish oil TG were converted into EE, which could easily be separated by molecular distillation, whereas the polyunsaturated fatty acids, including EPA and DHA, were concentrated into a mixture of residual 1(3)- and 2-monoacylglycerols (MG), 1,3- and 1,2(2,3)-diacylglycerols (DG), and TG, depending upon the extent of conversion. The process is demonstrated in Scheme 1. A similar alcoholysis reaction has been reported (19) where ethanol was employed as a solvent in the presence of up to 5% water, which resulted in large quantities of FFA due to hydrolytic side reactions. The plan was to employ molecular distillation to separate the volatile EE from the less volatile residual acylglycerol mixture, which subsequently can be converted into EE by chemical or lipase-catalyzed transesterification reactions and further concentrated. This is described in the accompanying paper (20).



SCHEME 1

EXPERIMENTAL PROCEDURES

The fungal lipase from *Mucor miehei,* provided as Lipozyme™, and the yeast lipase from *Candida antarctica,* provided as SP-435, were both supplied immobilized by Novo-Nordisk a.s. (Bagsvaerd, Denmark). Other lipases were supplied and employed directly as powder, without any preadjustment or optimization of pH, by Amano Enzyme Europe Ltd. (Milton Keynes, England), except the yeast lipase from *C. cylindracea* and the bacterial lipase from *Chromobacterium viscosum,* which were purchased from Sigma Chemical Company (St. Louis, MO). The activities stated for the *Pseudomonas fluorescens* lipase (PFL) and the *Pseudomonas* sp. lipase (PSL) were determined by the supplier in Amano lipase activity units, U/g, and are based on olive oil hydrolysis at 25°C. The fish oil triglycerides, originating from sardine oil, were provided by Pronova Biocare a.s. (Bergen, Norway), and contained 14.9% EPA and 9.8% DHA. They were used without further refinement. All solvents were of analytical grade and purchased from Merck AG (Darmstadt, Germany). Fatty acid analyses were performed on methyl esters in a Perkin-Elmer 8140 gas chromatograph (Beaconfield, Buckinghamshire, United Kingdom) with a 30-m capillary column, DB-225 30N 0.25 mm, with hydrogen as a carrier gas according to our previously described procedure (21). Preparative thin-layer chromatography was conducted on silica gel plates from Merck (Art. 5721) after washing with 50:50 mixture of chloroform methanol and heating at 110°C for 30 min. Elution was performed with 80:20:1 mixture of petroleum ether diethyl ether acetic acid. Rhodamine 6G (Merck) was used to visualize the bands, which subsequently were scraped off to methylate the fat as described above. Methyl esters of $C_{19:0}$ or $C_{21:0}$, purchased from Sigma, were added to the samples as internal standards before injection. When calculating the weight percentage, the type of ester moiety attached to the fatty acid was accounted for and corrected by calculated conversion parameters, from the gas chromatographic area percentages of the methyl esters. The parameters used were 1.201, 1.047, 0.996, and 1.047 for the MG, DG, TG, and EE, respectively.

Lipase-catalyzed ethanolysis. In a typical procedure, the lipase [0.5 g; PSL (Amano AK; 17,000 U/g) or PFL (Amano PS; 30,000 U/g)] was added as a powder to a mixture of the fish oil triglycerides (5.0 g, approximately 5.67 mmol) and absolute ethanol (0.80 g, 17.4 mmol). The resulting enzyme suspension was gently agitated at room temperature under nitrogen. After an appropriate time the reaction was discontinued by separating the enzyme by filtration.

RESULTS AND DISCUSSION

Nearly all commercially available lipases were screened in the described ethanolysis process, including the *M. miehei* (Novo), *C. antarctica* (Novo), *C. cylindracea* (Sigma), *P. fluorescens* (Amano PS), *Pseudomonas* sp. (Amano AK), *Geotrichum candidum* (Amano GC), *Penicillium roqueforti* (Amano R), *Aspergillus niger* (Amano A), *C. rugosa* (Amano AY), *Chromobacterium viscosum* (Sigma), *Humicula lanuginosa* (Amano CE), *Rhizopus delemar* (Amano D), *R. oryzae* (Amano F), *Penicillium camembertii* (Amano G), *C. lipolytica* (Amano L), *M. javanicus* (Amano M), and *R. niveus* (Amano N) lipases. Only a few lipases displayed activity under the chosen reaction conditions. The results firmly established that the bacterial *Pseudomonas* lipases from Amano, *P. fluorescens* lipase (PFL) and *Pseudomonas* sp. lipase (PSL), were superior in terms of fulfilling the high activity criteria toward saturated and monounsaturated fatty acids in fish oil, much lower activity toward EPA and DHA, and, at the same time, good tolerance toward the water-deficient alcoholic conditions. Other lipases

TABLE 1
The Results of Experiments with *Pseudomonas* Species Lipase at 20°C

	Time (h)					
Class[a]	1	2	4	8	13	24
Weight % of lipid classes						
EE	20.4	34.2	38.7	47.0	48.1	51.8
FFA	2.6	2.9	2.5	2.7	2.7	2.7
MG	5.0	9.4	13.0	17.9	16.8	15.9
DG	29.1	35.6	31.6	23.3	24.9	25.9
TG	42.9	17.9	14.2	9.1	7.5	3.7
Area % EPA						
EE	1.1	1.2	1.6	2.0	2.2	2.8
FFA	3.0	2.7	5.0	6.2	5.7	9.0
MG	12.0	12.8	16.4	19.2	22.5	26.1
DG	19.3	21.6	26.0	28.1	32.3	32.3
TG	19.2	23.2	30.3	31.8	32.7	32.0
MG/DG/TG	18.8	20.7	24.9	25.6	29.0	30.1
Area % DHA						
EE	1.5	1.4	1.9	2.4	2.8	3.6
FFA	2.5	2.7	4.9	6.4	6.7	9.9
MG	15.5	16.2	16.4	17.3	18.4	17.7
DG	12.7	13.2	13.7	14.1	15.1	14.9
TG	9.8	10.3	11.6	12.2	12.9	12.3
MG/DG/TG	11.3	12.8	13.8	14.9	15.9	15.7
Area % EPA + DHA						
EE	2.6	2.6	3.5	4.4	5.0	6.4
FFA	5.5	5.4	9.9	12.6	12.4	18.9
MG	27.5	29.0	32.8	36.5	40.9	43.8
DG	32.0	34.8	39.7	42.2	47.4	47.2
TG	29.0	33.5	41.9	44.0	45.6	44.3
MG/DG/TG	30.1	33.5	38.7	40.5	44.8	45.8
Weight % EPA						
EE	1.5	2.9	4.1	5.7	6.9	9.0
FFA	0.6	0.7	0.9	1.2	1.2	1.6
MG	3.5	7.8	12.1	21.4	21.8	22.4
DG	37.2	56.5	53.5	51.3	53.1	51.7
TG	57.3	32.2	29.4	20.3	17.0	15.4
MG/DG/TG	98.0	96.5	95.0	93.0	91.9	89.5
Weight % DHA						
EE	3.3	5.7	8.0	12.4	14.8	19.6
FFA	0.8	1.1	1.5	2.2	2.3	3.0
MG	7.5	15.6	22.8	33.0	29.9	26.2
DG	40.4	55.0	48.3	38.6	41.5	41.0
TG	48.1	22.6	19.4	13.8	11.4	10.3
MG/DG/TG	96.0	93.2	90.5	85.4	82.8	77.5

[a]Abbreviations: EE, ethyl esters; FFA, free fatty acids; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

TABLE 2
The Results of Experiments with *Pseudomonas fluorescens* Lipase at 20°C

	Time (h)						
Class[a]	1	2	4	8	13	24	49
Weight % of lipid classes							
EE	4.2	9.6	21.3	26.5	38.7	48.4	59.7
FFA	0.9	0.9	1.3	1.2	1.2	1.0	1.4
MG	2.2	2.9	4.8	10.2	15.1	18.1	16.7
DG	10.8	15.6	23.4	34.8	29.2	22.5	16.0
TG	81.9	71.0	49.1	27.3	15.8	9.9	6.3
Area % EPA							
EE	0.0	1.5	1.3	1.9	2.2	3.1	4.7
FFA	3.5	4.5	6.3	5.1	5.5	8.8	14.2
MG	11.6	12.7	13.7	12.7	14.5	16.9	21.5
DG	17.9	19.6	16.9	17.8	25.5	28.9	27.1
TG	15.9	16.4	18.0	22.0	29.2	31.8	26.7
MG/DG/TG	16.0	16.8	17.4	18.7	23.7	25.2	24.6
Area % DHA							
EE	0.0	1.3	1.1	1.5	2.0	2.7	3.7
FFA	3.4	3.5	4.3	4.4	3.2	9.4	8.4
MG	10.5	14.7	18.1	18.1	18.6	18.7	13.2
DG	12.2	12.9	13.5	10.9	13.8	14.4	11.8
TG	9.6	9.6	9.3	9.8	10.8	11.4	10.1
MG/DG/TG	9.9	10.3	11.1	11.5	14.2	15.4	12.1
Area % EPA + DHA							
EE	0.0	2.8	2.4	3.4	4.2	5.8	8.4
FFA	6.9	8.0	10.6	9.5	8.7	18.2	22.6
MG	22.1	27.4	31.8	30.8	33.1	35.6	34.7
DG	30.1	32.5	30.4	28.7	39.3	43.3	38.9
TG	25.5	26.0	27.3	31.8	40.0	43.2	36.8
MG/DG/TG	25.9	27.2	28.5	30.2	37.9	40.5	36.8
Weight % EPA							
EE	0.0	0.9	1.9	3.5	5.5	10.6	23.0
FFA	0.2	0.3	0.6	0.5	0.5	0.7	1.7
MG	1.4	2.0	4.1	8.0	12.7	18.8	25.5
DG	12.1	19.4	28.0	43.6	49.2	46.1	35.3
TG	86.3	77.5	65.4	44.4	32.2	23.7	14.4
MG/DG/TG	99.8	98.9	97.5	96.0	94.1	88.6	75.2
Weight % DHA							
EE	0.0	1.3	2.5	4.4	8.2	14.7	32.6
FFA	0.4	0.4	0.7	0.7	0.4	1.2	1.8
MG	2.0	3.8	8.5	18.5	27.1	33.7	28.1
DG	13.3	20.7	35.1	44.0	44.3	36.9	27.6
TG	84.3	73.9	53.2	32.5	19.9	13.5	9.8
MG/DG/TG	99.6	98.4	96.8	95.0	91.3	84.1	65.5

[a]For abbreviations see Table 1.

with good activity under these conditions included the immobilized *M. miehei* and *C. antarctica* lipases. The former lipase displayed a strong discrimination between EPA and DHA, which resulted in a high recovery of DHA but unacceptable losses of EPA. The *C. antarctica* lipase displayed high activity, but severe losses of both EPA and DHA were unavoidable. Both the *C. cylindracea* lipase from Sigma and the *P. roqueforti* lipase from Amano displayed activities that were too low for them to be utilized further. The performance of the former lipase depended upon addition of some water to the reaction medium.

Optimal conditions. Optimal results were obtained when the ethanolysis reaction was conducted at room temperature in the absence of any solvent with a stoichiometric amount of absolute ethanol. This means three molar equivalents of ethanol as based on glycerol or one equivalent as based on the number of ester functions were present in the glycerol backbone. With 10% dosage of lipase, as based on weight of the fish oil TG substrate, 50% conversion into EE was obtained in 24 h. In this reaction, the bulk of the saturated and monounsaturated fatty acids reacted, leaving the long-chain n-3 polyunsaturated fatty acids unreacted in the residual mixture, which consisted of MG, DG, and TG. Good insight into the performance of the lipases was provided by collecting samples while the ethanolysis reactions proceeded. Preparative thin-layer chromatography was applied to separate individual lipid classes, each of

which subsequently was methylated and subjected to gas–liquid chromatography (GLC) to reveal their fatty acid composition. Typical results are displayed in Tables 1 and 2 for PSL and PFL, respectively. The weight percentage of the produced EE was used directly to monitor the progress of the reaction in terms of conversion. Similarly, the weight percentage of the co-produced FFA was used as a measure to quantify the hydrolytic side reactions. The area percentage, provided by GLC fatty acid analysis of individual lipid classes, directly designates the extent of enrichment of EPA or DHA into individual lipid classes while the ethanolysis reaction proceeded. Finally, the weight percentage of EPA or DHA denotes the proportion of their total weight in the initial fish oil and can be defined as their recovery or yield. Thus, the weight percentage of EPA and DHA in the combined MG, DG, and TG was used to define the recovery of both EPA and DHA in the concentrate.

As shown in Table 1 for the ethanolysis reaction with PSL, more than 50% conversion into EE was obtained after 24 h. The acylglycerol mixture comprised 30% EPA and 16% DHA, i.e., 46% EPA + DHA. The recovery of DHA and EPA in particular was high indeed, over 80% for DHA and higher than 90% for EPA. The reversed fatty acid selectivity favoring DHA, which diverges from the most common behavior of lipases to discriminate between EPA and DHA in favor of EPA, is noticeable and most unusual (*vide infra*). The extent of the hydrolytic side reactions, as measured by the amount of FFA present in the reaction mixture, remained below 3% throughout the reaction. Although both PSL and PFL fulfilled the previously mentioned criteria excellently, there remained some significant differences between them as evidenced by comparing Tables 1 and 2. The rate of the PSL-promoted reaction was considerably faster despite lower initial activity in terms of lipase activity units. The EPA and DHA recovery remained lower for PFL, as did their area percentage levels. On the other hand, the extent of hydrolytic side reactions remained more favorable for PFL. Therefore, PSL was superior to PFL in terms of fulfilling the requirements for the lipase, and the discussion below will mostly be confined to PSL.

Lipid class composition. The results indicate that an equilibrium in terms of lipid class composition had been obtained after 24 h at 52% conversion. This is clearly evident from the weight percentage part of Table 1, which displays the individual lipid class composition as the reaction proceeded. This, however, by no means indicates that an equilibrium in terms of fatty acid composition of each class had been reached. The extent of the hydrolytic side reactions remained constant throughout the reaction. This seems to establish that the hydrolysis process is considerably faster than the reverse esterification processes, which is general behavior of lipases and other hydrolytic enzymes (22).

The DG content reached a maximum of 36% after 2 h reaction time, when it dominated in the mixture. As the reaction proceeded, its content gradually declined to the 25% equilibrium level. The MG content, on the other hand, gradually increased to the 18% level after 8 h and remained close to that level. This behavior implies that DG are inferior substrates for the lipase as compared to MG under these conditions.

Area percentage of EPA and DHA. The area percentage directly designates the extent of enrichment of EPA or DHA into individual fractions or lipid classes. Thus, the area percentage of the acylglycerol product mixture (MG/DG/TG) is a measure of the composition of the concentrate that is obtained after separating the EE and FFA. It does not, however, indicate the recovery of EPA and DHA from the initial oil into that fraction, which is expressed by the weight percentage. Table 1 shows that, during the progress of the reaction, the DG and TG fractions share the highest composition (32–33%) of EPA, while the MG fraction is considerably lower but constantly increasing in EPA all the way through to reach a maximum (26%) after the reaction had proceeded toward the equilibrium level in terms of conversion.

DHA behaved quite differently in the sense that the MG fraction was the most highly enriched with DHA (18%) throughout the reaction.

EPA and DHA recovery. The weight percentage denotes the proportion of the EPA and DHA content in individual fractions during progress of the reaction as based on their total content in the initial fish oil. It is directly affected by both the quantity of the fraction and its EPA or DHA content. The weight percentage of EPA and DHA in the combined acylglycerols is a direct measure of their recovery or yield into the acylglycerol mixture from which the concentrate is derived. The weight percentage distribution of EPA and DHA into individual MG, DG, and TG as well as EE and FFA classes with progress of the reaction is displayed in the weight percentage part of Table 1.

All processes involving DHA proceeded faster than those for EPA. As has been pointed out above, this behavior is most unusual because most lipases display a higher activity toward EPA than DHA. This has been attributed to the location of the carbon-carbon bond closest to the carboxyl group, one C-C bond closer in DHA than EPA, adding to the strain of the enzyme-active site (23,24). As far as we know, this selectivity in favor of DHA has never before been reported for a lipase. Similar behavior was retained in our preliminary hydrolysis studies of fish oil EE under aqueous conditions for both PSL and PFL, which indicates that this unusual selectivity is a feature of these lipases, independent of the reaction conditions and type of reaction. In the studies of Zuyi and Ward (19) of fish oil with these lipases, similar behavior can be noticed from their results, but it was not specifically pointed out by these authors.

At 48% conversion after 13 h reaction time, the recoveries of EPA and DHA were already above the previously set criteria of 90% EPA and 80% DHA. At that stage, the combined area percentage of EPA and DHA in the acylglycerol mixture had reached 45%, which also fulfills the criterion for EPA + DHA composition. At 52% conversion after 24 h, the recovery had dropped slightly for EPA, but more for DHA. At that stage, an equilibrium in terms of conversion and lipid class composition had been reached, and reaction beyond that re-

sulted in a slight increase in EPA + DHA composition but a considerable drop in EPA and DHA recovery.

Water content control. When an enzymatic reaction is allowed to proceed in an organic medium, the water present in the reaction system plays a crucial role for maintaining optimal activity of the lipase (22,25,26). Some water is always required for lipases to retain their activity, but the amount of water required appears to vary considerably among different lipases. It also depends upon the reaction medium, polarity of organic solvents, etc. A reaction that is conducted in a medium containing less than 1% water is usually considered to be under anhydrous conditions. Often, much less water is required for lipases to display optimal performance.

For the lipase-promoted type of transformations described in this report, complete control of the water content is important to ensure consistent results in terms of both activity and the consequential hydrolytic side reactions, which must be kept at a minimum. This means that a compromise is required between maximum activity and hydrolysis. In the reactions involving PSL and PFL, no extra water was added to the reaction medium, which means that the water content was far below the 1% criterion for anhydrous conditions.

Lipase immobilization. The employment of lipase powder was problematic when it came to reusing the powder, and satisfactory productivity of the lipases was rather obscure. When the reactions were repeated with recovered lipase, dramatic drops in activity were noticed. All attempts to reestablish the initial activity, by adding water to compensate for the hydrolytic side reactions, failed and resulted in aggregation of the lipase powder. This appears to limit the exposure of the enzyme surface to the substrate molecules. To solve that problem, immobilization (22,27) of the lipase on a support material had to be considered. The highly successful immobilization of PSL and PFL on Duolite resin and Amberlite adsorbent will be described in a separate publication.

Compromise and control. When utilizing the residual acylglycerol mixture from the enzymatic ethanolysis reaction as an intermediary material for further concentration of EPA and DHA, the EPA and DHA area percentage composition of that mixture is clearly important. Of no less concern is the recovery of EPA and DHA into that fraction as expressed directly by their weight percentages in the glyceride mixture. The results presented in this report clearly indicate that a compromise is needed in this respect of maximizing EPA and DHA composition and minimizing losses of EPA and DHA. The results appear to suggest that, among the various parameters involved, the initial composition of the feed oil and the extent of conversion up to the equilibrium stage, but not beyond that, may be the most eminent factors in this respect. The interrelationship between these factors and the various parameters involved in the reaction, such as dosage of lipase, water content, lipase activity, reaction time, equilibrium in terms of lipid class composition, and EPA and DHA composition of each lipid constituent, is therefore of obvious importance. The reaction should not be allowed to proceed beyond an equilibrium in terms of lipid class composition, because undesired fatty acid interchange processes between the various lipid constituents then start to dominate. The task therefore has features quite common to the classical kinetic resolution (28), and the same theories apply here. What adds to the complexity of the task is the fact that three fatty acids are attached to the same glycerol backbone more or less randomly, adding complications related to different selectivities of the enzyme being used toward the different acylglycerols as well as its regioselectivity.

ACKNOWLEDGEMENTS

The work described in this report was carried out in collaboration with Pronova Biocare and Norsk Hydro in Norway, whose financial support is acknowledged. T.T. Hansen at Novo-Nordisk and Amano Enzyme Europe Ltd. are acknowledged for enzymes.

REFERENCES

1. Nelson, G.J. (ed.), *Health Effects of Dietary Fatty Acids,* American Oil Chemists' Society, Champaign, 1991.
2. Haraldsson, G.G., and B. Hjaltason, Using Biotechnology to Modify Marine Lipids, *INFORM 3:*626–629 (1992).
3. Haraldsson, G.G., The Application of Lipases for Modification of Fats and Oils, Including Marine Oils, in *Advances in Fisheries Technology for Increased Profitability,* edited by M.N. Voigt and R. Bhotta, Technomic Publishing Co., Inc., Lancaster, Pennsylvania, 1989, pp. 337–357.
4. Maehr, H., G. Zenchoff, and D.L. Coffen, Enzymatic Enhancement of n-3 Fatty Acid Content in Fish Oils, *J. Am. Oil Chem. Soc. 71:*464–467 (1994).
5. Lie, Ö., and G. Lambertsen, Fatty Acid Specificity of *Candida cylindracea* Lipase, *Fette, Seifen, Anstrichm. 88:*365–367 (1986).
6. Shimada, Y., K. Maruyama, M. Nakamura, S. Nakayama, A. Shugihara, and Y. Tominaga, Selective Hydrolysis of Polyunsaturated Fatty Acid-Containing Oil with *Geotrichum candidum* Lipase, *J. Am. Oil Chem. Soc. 72:*1577–1581 (1995).
7. Shimada, Y., K. Maruyama, S. Okazaki, M. Nakamura, A. Sugihara, and Y. Tominaga, Enrichment of Polyunsaturated Fatty Acids with *Geotrichum candidum* Lipase, *Ibid. 71:*951–954 (1994).
8. Tanaka, Y., J. Hirano, and T. Funada, Concentration of Docosahexaenoic Acid in Glyceride by Hydrolysis of Fish Oil with *Candida cylindracea* Lipase, *Ibid. 69:*1210–1214 (1992).
9. McNeill, G.P., R.G. Ackman, and S.R. Moore, Lipase-Catalyzed Enrichment of Long-Chain Polyunsaturated Fatty Acids, *Ibid. 73:*1403–1407 (1996).
10. Moore, S.R., and G.P. McNeill, Production of Triglycerides Enriched in Long-Chain n-3 Polyunsaturated Fatty Acids from Fish Oil, *Ibid. 73:*1409–1414 (1996).
11. Haraldsson, G.G., P.A. Höskuldsson, S.Th. Sigurdsson, F. Thorsteinsson, and S. Gudbjarnason, The Preparation of Triglycerides Highly Enriched with ω-3 Polyunsaturated Fatty Acids *via* Lipase-Catalyzed Interesterification, *Tetrahedron Lett. 30:*1671–1674 (1989).
12. Yamane, T., T. Suzuki, Y. Sahashi, L. Vikersveen, and T. Hoshino, Production of n-3 Polyunsaturated Fatty Acid-Enriched Fish Oil by Lipase-Catalyzed Acidolysis Without Solvent, *J. Am. Oil Chem. Soc. 69:*1104–1107 (1992).
13. Haraldsson, G.G., B.Ö. Gudmundsson, and Ö. Almarsson, The Synthesis of Homogeneous Triglycerides of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase, *Tetrahedron 51:*941–952 (1995).
14. Shimada, Y., A. Sugihara, H. Nakano, T. Kuramoto, T. Nagao,

M. Gemba, and Y. Tominaga, Purification of Docosahexaenoic Acid by Selective Esterification of Fatty Acids from Tuna Oil with *Rhizopus delemar* Lipase, *J. Am. Oil Chem. Soc. 74:*97–101 (1997).
15. Hills, M.J., I. Kiewitt, and K. Mukherjee, Enzymatic Fractionation of Fatty Acids: Enrichment of γ-Linolenic Acid and Docosahexaenoic Acid by Selective Esterification Catalyzed by Lipases, *Ibid. 67:*561–564 (1990).
16. Pedersen, S.B., and G. Hölmer, Studies of the Fatty Acid Specificity of the Lipase from *Rhizomucor miehei* Toward 20:1n-9, 20:5n-3, 22:1n-9 and 22:6n-3, *Ibid. 72:*239–243 (1995).
17. Langholz, P., P. Andersen, T. Forskov, and W. Schmidtsdorff, Application of a Specificity of *Mucor miehei* Lipase to Concentrate Docosahexaenoic Acid (DHA), *Ibid. 66:*1120–1123 (1989).
18. Takagi, T.J., Fractionation of Polyenoic Acids from Marine Lipids, *Ibid. 66:*488–489 (1989).
19. Zuyi, L., and O.P. Ward, Lipase-Catalyzed Alcoholysis to Concentrate the n-3 Polyunsaturated Fatty Acid of Cod Liver Oil, *Enzyme Microb. Technol. 15:*601–606 (1993).
20. Breivik, H., G.G. Haraldsson, and B. Kristinsson, The Preparation of Highly Purified Concentrates of EPA and DHA, *J. Am. Oil Chem. Soc. 74:*1425–1429 (1997).
21. Haraldsson, G.G., and Ö. Almarsson, Studies on the Positional Specificity of Lipase from *Mucor miehei* During Interesterification Reactions of Cod Liver Oil with n-3 Polyunsaturated Fatty Acid and Ethyl Ester Concentrates, *Acta Chem. Scand. 45:*723–730 (1991).
22. Haraldsson, G.G., The Application of Lipases in Organic Synthesis, in *The Chemistry of the Functional Groups, Supplement B2: The Chemistry of Acid Derivatives,* edited by S. Patai, John Wiley & Sons, Chichester, 1992, Vol. 2, pp. 1395–1473.
23. Haraldsson, G.G., The Preparation of Triglycerides Highly Enriched with n-3 Polyunsaturated Fatty Acids *via* Lipase-Catalyzed Interesterification, in *Proceedings of the 15th Scandinavian Symposium on Lipids,* edited by V.K.S. Shukla and G. Hölmer, Lipidforum, Gothenburg, Sweden, 1989, pp. 245–248.
24. Miller, C., H. Austin, L. Posorske, and J. Gonzlez, Characteristics of an Immobilized Lipase for the Commercial Synthesis of Esters, *J. Am. Oil Chem. Soc. 65:*927–931 (1988).
25. Dordick, J.S., Enzymatic Catalysis in Monophasic Organic Solvents, *Enzym. Microb. Technol. 11:*194–211 (1989).
26. Koskinen, A.M.P., and A.M. Klibanov (eds.), *Enzymatic Reactions in Organic Media,* Blackie Academic and Professional, London, 1996.
27. Klibanov, A.M., Immobilized Enzymes and Cells as Practical Catalysts, *Science 219:*722–727 (1983).
28. Kagan, H.B., and J.C. Fiaud, Kinetic Resolution, in *Topics in Stereochemistry,* edited by E.L. Eliel and S.H. Wilen, Wiley-Interscience, New York, 1988, Vol. 18, pp. 249–330.