Application of a Specificity of *Mucor miehei* Lipase to Concentrate Docosahexaenoic Acid (DHA)

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Lipases catalyze the reesterification or hydrolysis of fats and oils and may show specificity towards certain fatty acids. An immobilized lipase from the fungus *Mucor miehei* shows specificity towards docosahexaenoic acid (DHA) (DHA being a poor substrate), whether the DHA is used as a free fatty acid (FFA) as substrate in esterification with methanol, or fatty acid methyl ester (FAME) is used as a substrate for hydrolysis. The specificity of the lipase from *M. miehei* may be applied to concentrate DHA originating from a marine oil in either the FAME or the FFA fraction, which can be separated.

Eicosapentaenoic acid (EPA, C 20:5 n-3) and docosahexaenoic acid (DHA, C 22:6 n-3) have been shown to have positive preventive health effects on people with different cardiovascular disorders, such as arteriosclerosis and myocardiac infarct (1). Physiological studies suggest that some of the effect is derived from competitive action between arachidonic acid (AA, C 20:4n-6) and EPA on the enzyme cyclooxygenase. Cyclooxygenase catalyzes the first step in the formation of the transmitter compounds, prostaglandins and thromboxanes. DHA is found in high amounts in the phospholipids of the human brain, retina and testis. The physiological effect of DHA is not known, but EPA and DHA can be converted to each other *in vivo*, that is, DHA can serve as a pool of EPA. The main source of EPA and DHA is marine foods.

Saturated fatty acids are generally believed to have an adverse effect on cardiovascular disorders. Therefore, we have attempted to concentrate these n-3 fatty acids and take greater advantage of their potential health benefits. In many fish oils (triglycerides), the n-3 fatty acid is placed in the 2-position of the triglyceride surrounded by saturated and monounsaturated acids (2). This fact limits the possibilities of separation by simple crystallization.

Fatty acids can be separated using preparative HPLC (3) either employing the free acids, or their clathrates with urea (4). Enzymatic concentration of the acids from cod roe phospholipids has also been demonstrated, using phospholipase A_2 (5). A similar approach could be employed with triglycerides from fish oil, using a 1,3-specific lipase, leaving behind 2-monoglycerides with a high content of EPA and DHA. Initial attempts to accomplish this failed due to acyl migration from the 2-position to the 1- or 3-position (unpublished results).

Lipases catalyse esterification, hydrolysis or exchange of fatty acids in esters, depending on the direction of reaction favored by the experimental conditions (concentration of fatty acids, esters, water and alcohol). Specific lipases give triglycerides, which are unobtainable by simple chemical interesterification methods (6), (7), (8), (9). Using a 1,3-specific lipase, a specific fatty acid can become incorporated into the outer position without changing the fatty acid residues in the center position by a process known as acidolysis. In addition to specificity towards certain positions in triglycerides, lipases may show specificity towards certain fatty acids. In the present studies we employed a commercial immobilized 1,3-specific lipase from the fungus *Mucor Miehei* (10).

MATERIALS AND METHODS

Substrates. Refined Sand Eel oil (fish oil produced from *Ammodytes sp.*) was applied to produce FAMEs and FFAs, as well as concentrates of PUFAs (Fatty acid composition, see Table 1). FFA from this oil (low content of EPA and DHA) was prepared using sodium hydroxide in 50% ethanol (FFA_{se}). Methyl esters from this oil (low content of EPA and DHA) were prepared using NaOH in methanol ($FAME_{se}$).

Concentration of EPA and DHA was carried out as described by Haagsma *et al.* (4). The concentrate consists of 44% FFA and 56% FAME. The concentrate was applied for a lipase catalyzed reaction between FFA and FAME. (Fatty acid composition see Table 1). A concentrate was applied to produce FAME with a high content of EPA and DHA ($FAME_c$). This was done by using metallic sodium in methanol. (Fatty acid composition of the concentrate, see Table 1) — enzyme: Lipozyme IM 20 from NOVO Industri A/S, Copenhagen, Denmark; declared activity 23.2 BIU/*g*.

METHODS

Acidolysis was carried out in 12 ml hexane containing 400 mg Sand Eel oil and 820 mg FFA + FAME made

TABLE 1

Fatty Acid Composition of Refined Sand Eel Oil, and Concentrate Made by Urea Inclusion.

Fatty acid	Original oil (%)	Urea concentrate (%)
C 14:0	6.7	—*
C 16:0	13.0	—*
C 16:1	5.8	2.5
C 18:0	—*	—*
C 18:1	5.6	—*
C 18:2	—*	—*
C 18:3	—*	—*
C 20:1	10.6	2.2
C 18:4	5.6	11.0
C 20:3	—*	2.3
C 22:1	17.8	—*
C 20:5	10.6	27.1
C 22:5	—*	3.9
C 22:6	8.2	27.2

*Less than 2%.

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by urea inclusion (5), to which was added 300 mg of immobilized *M. miehei* lipase. The results are the average of three determinations. Enzymatic hydrolysis of $FAME_{se}$ with a low content of EPA and DHA was carried out in a 12 ml hexane solution containing 325 mg of $FAME_{se}$ and 300 mg of lipase. The experiments were performed at room temperature in a tube that was rotated "end over end." The reaction was terminated by filtering off the lipase (triplicate). Hydrolysis of $FAME_c$ with a high content of EPA and DHA was carried out with 900 mg of $FAME_c$; other conditions as above. In reaction between FFA and FAME, 820 mg of mixed substrates (FFA+FAME) were used; other conditions as above. Esterification of FFA_{se} was done with 900 mg FFA_{se} in a mixture of 11 ml hexane and 1 ml methanol. The enzyme dose was 1200 mg; other conditions as above.

Methods of analysis. FFA and FAME were separated on 0.5 mm TLC plates made of Kieselgel (Silica gel) G 60 (Merck, Darmstadt, W. Germany). The eluents were hexane:diethylether:acetic acid 80:20:2 v/v/v. The plates were developed by 2,7-dichlorfluorescein (0.2 % in 96 % ethanol). The bands of FFA and FAME were scraped off and prepared for GC analysis. Methylation for GC analysis was done by boiling in methanol under reflux with boron trifluoride as the catalyst. Gas chromatography on the methyl esters was performed on a HP 5890 gas chromatograph (Hewlett Packard, Avondale, Pennsylvania), on a 30 m long, 0.75 mm inner diameter, SP 2330 open tubular column (Supelco Inc. Bellefonte, Pennsylvania); the carrier gas was nitrogen. The relative content of a fatty acid in the FFAs or FAMEs is expressed as weight percent of the total amount of fatty acids in the FFAs or the FAMEs (weight percent fatty acid approximated by the area percent of the detector response).

RESULTS AND DISCUSSION

During the investigation of the incorporation of EPA and DHA into triglycerides, we observed an increase in the concentration of DHA in the fatty acid fraction. The enzymatic incorporation of EPA and DHA was carried out by acidolysis using Sand Eel oil and urea concentrate, which consists of FFA and FAME. The acidolysis was catalyzed by lipase from *Mucor miehei.* Unexpectedly, we observed that the content of DHA in the FFA was increasing very markedly during acidolysis. As the acidolysis is a complex set of reactions we chose to carry out some simpler reactions to clarify the cause of the high content of DHA obtained in the FFAs during acidolysis. To do this the hydrolysis/esterification reaction between FFA and methanol under enzyme catalysis was investigated:

$$\text{FFA} + \text{MeOH} \overset{\text{lipase}}{\leftrightarrow} \text{FAME} + H_2O$$

Reaction between FFA and FAME. During a lipase catalyzed reaction in a mixture of FAME and FFA, the content of FFA and FAME being essentially equal, the weight percent DHA in the FFAs changed as a function of time as shown in Figure 2.

As a consequence of the reaction the content of FFA decreased and that of FAME increased (data not shown). During the reaction between FFA and FAME the content of DHA in the FFAs becomes very high. This is a manifestation of the specificity towards DHA and also a result of the equilibrium being displaced towards FAME. DHA remained, to a higher degree than the other fatty acids, as a FFA.

Hydrolysis of FAMEs. During the hydrolysis of FAMEs with a high, as well as a low EPA and DHA content, it was demonstrated that in the beginning the DHA content in the FFAs was small, after which it gradually approached the level of DHA in the FAMEs before hydrolysis (Table 1). During hydrolysis of $FAME_c$s with a high content of DHA, the course of DHA concentration as a function of time is shown in Figure 3. During hydrolysis of $FAME_{se}$s low in DHA content the normalized concentration factor F_i of dif-

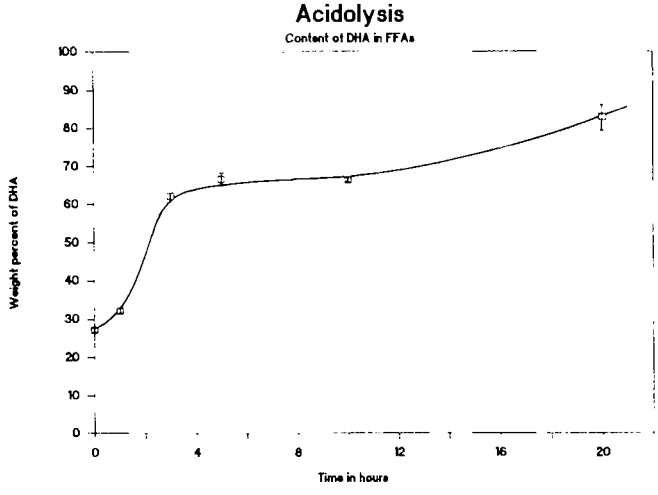

FIG. 1. Relative content of DHA in the FFA during acidolysis as a function of the time of reaction. Standard deviations indicated (the vertical bars show 2 times the standard deviation).

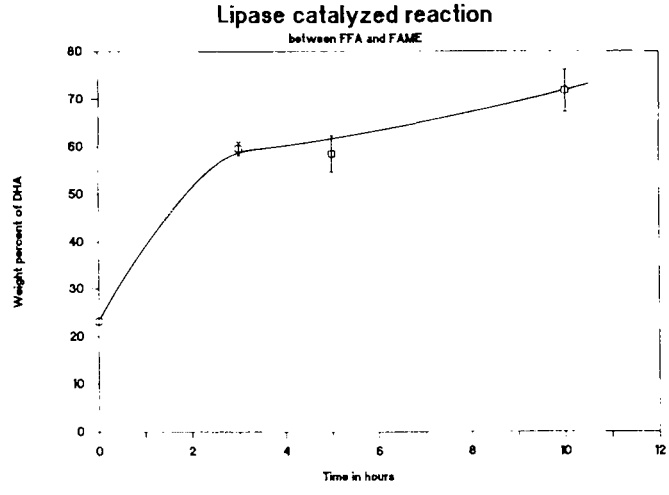

FIG. 2. Relative DHA content in the FFAs as a function of time during lipase catalyzed reaction between FFA and FAME. Standard deviations indicated.

ferent fatty acids was a function of time as will be shown in Figure 4. The concentration factor f_i is defined as:

$$f_i = \frac{FFA_i}{FAME_i}$$

FFA_i is the content of a fatty acid in the FFAs after a certain time of reaction. $FAME_i$ is the content of the methyl ester of the fatty acid in the original FAMEs. A concentration factor (f_i) is normalized in this context by dividing it by the concentration factor f_{DHA} of DHA:

$$F_i = \frac{f_i}{f_{DHA}} = \frac{(FFA_i/FAME_i)}{(FFA_{DHA}/FAME_{DHA})} = \frac{(FFA_i/FFA_{DHA})}{(FAME_i/FAME_{DHA})}$$

As the time of reaction increases, the normalized concentration factor will approach the value 1 as a consequence of the composition of the FFAs and FAMEs approaching that of the original FAMEs:

$$Fi = \frac{(FFA_i/FFA_{DHA})}{(FAME_i/FAME_{DHA})} \rightarrow \frac{(FAME_i/FAME_{DHA})}{(FAME_i/FAME_{DHA})} = 1 \text{ as } t \rightarrow \infty$$

It is presumed that the value of the equilibrium constant is the same for all of the fatty acids. It can be seen from Figure 4 that in the beginning of the hydrolysis all other fatty acids except DHA have a concentration factor greater than that of DHA. That is, the non-DHA FAMEs are being hydrolyzed faster than methyl esters of DHA. The fatty acids shown are those present in the original FAME in appreciable amounts. Figure 4 shows that the normalized concentration factor of EPA has approximately the same course as the other fatty acids, yet the concentration factor of EPA is somewhat smaller. In the beginning of the hydrolysis EPA is concentrated in the FFAs, but to a smaller degree than the other FAMEs. Methyl esters of DHA are concentrated in the FAMEs.

Esterification of FFA_{se} by MeOH. The content of DHA in the FFA during a lipase catalyzed esterification of FFA_{se} by MeOH is shown as a function of time in Figure 5. During esterification of FFA_{se} by MeOH, DHA was concentrated in the FFAs. This again shows DHA to be a poor substrate for the lipase as the esterification of DHA is not being catalysed by the lipase. By esterification of FFA_{se}s from Sand Eel oil by MeOH, we have achieved 48% DHA (Figure 5) in the FFAs, and by the reaction between FFA and FAME we achieved 72% DHA (Figure 2) in the FFAs. The esterification reaction may become economically attractive because the important fatty acids, EPA and DHA, normally only can be produced from marine oils. The oils must be hydrolyzed to form FFA. Following the lipase catalyzed esterification, the FFAs and the FAMEs must be separated. The FFAs pro-

TABLE 2

The DHA Content in Weight Percent in the FFAs as a Function of Time During Hydrolysis of FAMEs with a Low and a High DHA Content.

	DHA content	
% FAME	% FFA 1 h	% FFA 20 h
8.2	1.2	5.4*
20.7	7.4	18.3

*17 hours.

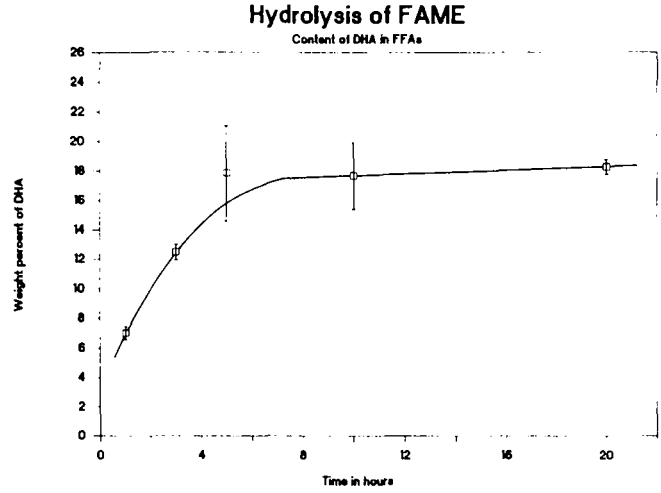

FIG. 3. The content of DHA (as weight percent) in the FFA as a function of time during hydrolysis of methyl esters ($FAME_c$) with a high content of DHA. Standard deviations indicated.

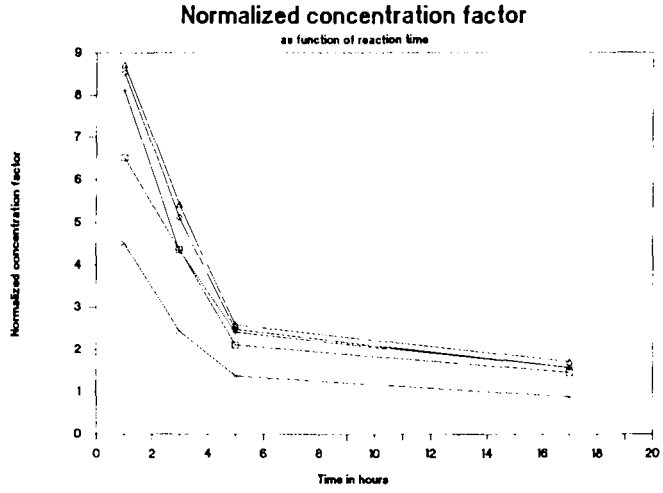

FIG. 4. Normalized concentration factors as a function of the time of reaction during hydrolysis of $FAME_{se}$ derived from sand eel oil ∇ C 14:0, × C 16:0, ◇ C 20:1, △ C 22:1, + C18:4, □ C 20:5.

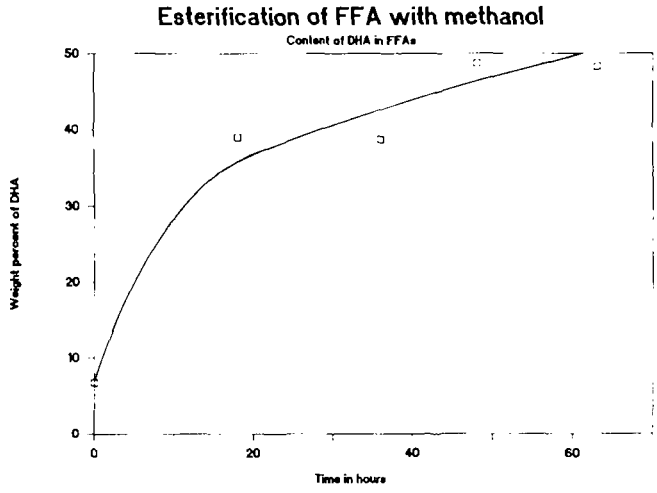

FIG. 5. Relative content of DHA in the FFAs as a function of the time of reaction during a lipase catalyzed esterification of FFA_{se} by methanol.

duced can be used directly or they can be applied in interesterification reactions for e.g. triglycerides (acidolysis).

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