0273-2289/97/6803—0171$12.00

Enzymatic Hydrolysis of Anchovy Oil: Production of Glycerides Enriched in Polyunsaturated Fatty Acids

G. ÜSTÜN,* S. GÜNER, G. ARER, S. TÜRKAY, AND A. T. ERCIYES

Istanbul Technical University, Faculty of Chemistry-Metallurgy, Chemical Engineering Department 80626 Maslak, Istanbul, Turkey

Received January 22, 1996; Accepted March 26, 1997

ABSTRACT

In an attempt to produce the polyunsaturated fatty acid (PUFA)-enriched glycerides, commercially available Turkish anchovy oil (PUFA content of 27%), was hydrolyzed with 1,3-specific *Rhizomucor miehei* lipase. After the hydrolysis, the triglyceride (TG), diglyceride (DG), monoglyceride (MG), and free fatty acid (FFA) composition of the reaction mixture was determined, and fatty acid components of these fractions were analyzed. *R. miehei* lipase released PUFA extremely slowly, resulting in their accumulation in the TG and DG fractions, especially in TG. The PUFA content in the glyceride mixture (including TG, DG, and MG) increased as hydrolysis progressed. The effects of operational parameters (pH, temperature, time, and enzyme concentration) on the extent of hydrolysis were investigated. Based on these results, optimal reaction conditions were established. At optimal conditions (pH 4.0, 35°C, 3 h, and enzyme concentration of 500 U/g oil), the level of PUFA in the glyceride mixture was raised to 40%. The individual TG and DG fractions contained 45 and 30% PUFA, respectively. Less than 2% of the total PUFA was lost in the FFA fraction.

Index Entries: Anchovy oil; hydrolysis; *Rhizomucor miehei*; lipase; polyunsaturated fatty acid (PUFA).

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Since the pioneering epidemiological work of Dyerberg and Bang *(1)* on Greenland Eskimos suggested a possible link between low incidence of heart diseases and the consumption of sea foods, many studies have been published on the role of n-3 polyunsaturated fatty acids (PUFAs) in human health and diseases. These fatty acids, especially eicosapentaenoic acid (20:5 n-3 or EPA) and docosahexaenoic acid (22:6 n-3 or DHA), have been reported to have beneficial effects in cardiovascular diseases, autoimmune disorders, and other inflammations *(2–5)*.

Marine lipids, such as fish oils, are the main source of n-3 PUFA. In literature, a number of methods have been proposed and employed for preparing n-3 PUFA-enriched fatty acids or their methyl (or ethyl) esters from fish oils, including urea adduct formation *(6,7)*; chromatographic separation by HPLC *(8,9)*; silver resin chromatography *(10)* or adsorption chromatography *(11,12)*; and supercritical fluid extraction *(13,14)*.

However, it has also been reported that EPA and DHA ethyl (or methyl) esters are not as easily incorporated into plasma TG as EPA and DHA in the TG form *(15)*. Furthermore, triglycerides are sometimes preferred for delivery of these PUFA to human subjects, in order to avoid complications caused by production of toxic methanol or less toxic ethanol *(13)*. Therefore, it is important to develop purification and concentration methods for PUFA in the glyceride form.

Recently, it was stated that several lipase-catalyzed reactions have been studied to concentrate the PUFA in the glyceride form *(16–21)*. Cod liver oil has been enriched to a level of above 60–65% n-3 PUFA by interesterification with an immobilized lipase from *Rhizomucor miehei (16)*. *Candida cylindracea (17,18)* and *Aspergillus niger (19)* lipases have been used for producing PUFA-rich glycerides from tuna oil and sardine oil, respectively. *Chromobacterium viscosum (20)* and *Pseudomonas* sp. *(21)* lipases have been tested to concentrate EPA and DHA in sardine oil by acidolysis or hydrolysis reactions. Commercially available menhaden and anchovy oils with n-3 PUFA contents of 29 and 34%, respectively, were converted enzymically with Amano P (*Pseudomonas sp.*) lipase in hexane, to mixtures of glycerides with n-3 PUFA contents of approx 50% *(22)*.

In Turkey, the beneficial effects of n-3 PUFA have also been divulged; however, no record of study on fish oils has been encountered in the scientific literature of Turkey. Therefore, we have attempted to produce enzymatically n-3 PUFA-enriched glycerides from Turkish fish oils for food and pharmaceutical uses.

Commercially available Turkish anchovy oil, with annual production of around 20,000 t, was used as substrate, and it was hydrolyzed with 1,3-specific *R. miehei* lipase, producing PUFA-rich TG and DG. To find the

optimum operating conditions, the effects of hydrolysis parameters (pH, temperature, time, and enzyme concentration) on the extent of hydrolysis and on the concentration of PUFA in the glyceride fractions were investigated. The usefulness of the *R. miehei* lipase is also discussed in this respect.

MATERIALS AND METHODS

Materials

Anchovy oil was obtained from Trabzon Fish Oil and Meal Factory (Trabzon, Turkey) and stored under nitrogen gas atmosphere to prevent oxidation. The same batch of anchovy oil was used throughout this investigation. *R. miehei* (Lipozyme 10,000 L) lipase, with an activity of 10,000 U/g, was provided by Novo-Nordisk A/S (Copenhagen, Denmark). Other reagents were analytical grade and purchased from Merck (Darmstadt, Germany).

Analysis of Anchovy Oil

Anchovy oil was analyzed for acid, saponification, and iodine values, unsaponifiable matter content, and density and refractive index, according to standard AOCS methods *(23)*. The experimental results are given in Table 1.

The triglyceride (TG), diglyceride (DG), monoglyceride (MG), and free fatty acid (FFA) composition of anchovy oil was determined by a thin-layer chromatography/flame-ionization detector (TLC/FID) analyzer (Iatroscan TH-10; Iatron, Tokyo, Japan), according to the method of Dandik and coworkers *(24)*. Since Turkish anchovy oil consisted of 98.74% TG, 0.11% DG, 0.17% MG, and 0.98% FFA, it was assumed to be 100% TG throughout this investigation.

For the determination of fatty acid composition, the oil was esterified with borontrifluoride–methanol agent and analyzed by capillary gas chromatography in a Hewlett-Packard 5890 Series 2 apparatus (Hewlett-Packard, Waldron, Germany) fitted with a FID and a data processor, under the following conditions: column, Ultra 2 (25 m × 0.32 mm) with 0.52 μm film thickness of 5% diphenyl, 95% dimethyl polysiloxane; nitrogen carrier gas at 1.72 mL/min; air flow of 450 mL/min; hydrogen flow of 69 mL/min; injection, split 50:1, 0.5 μL; injector temperature, 225°C; flame ionization detector temperature, 250°C; and oven temperature program, 150°C (5 min), 150–225° (5°C/min), 225°C (30 min). Peaks were identified by comparing the retention times with those of a mixture of standard methyl esters. Menhaden oil fatty acid methyl esters (Sigma, Poole, UK) were also utilized as secondary standard.

Table 1
Main Oil Characteristics of Turkish Anchovy Oil

Acid value	2.4
Saponification value	206.2
Iodine value	152.1
Unsaponifiable matter (wt%)	1.4
Density, 20°C (g/mL)	0.9140
Refractive index, 20°C	1.4785

Table 2
Fatty Acid Components of Turkish Anchovy Oil

Fatty acid components	wt % of total fatty acids
14:0	6.5
15:0	0.3
16:0	19.4
16:1	9.0
16:2	0.7
16:3	0.2
16:4	0.9
17:0	0.8
18:0	4.2
18:1	22.0
18:2	2.8
18:3	1.8
18:4	1.3
20:0	0.2
20:1	0.9
20:2	2.0
20:4	1.4
20:5 (EPA)	7.6
22:1	2.1
22:4	1.6
22:5	1.6
22:6 (DHA)	12.7

The fatty acid composition of Turkish anchovy oil is summarized in Table 2.

Enzymatic Hydrolysis of Anchovy Oil

The hydrolysis reaction was carried out in a 250-mL four-necked flask equipped with a stirrer, a temperature controller, and an inert gas connec-

tion. Twenty-five mL (23 g) anchovy oil and 25 mL buffer (0.1 *M* citric acid–0.23 *M* sodium dihydrogen phosphate) containing the proper amount of enzyme were placed into the reaction vessel and stirred at constant temperature for a prescribed period under nitrogen gas atmosphere to prevent oxidation. The stirring rate was adjusted to 7*g* in all experiments.

The reactions were monitored by TLC/FID analysis of the reaction products. Samples of 1 mL were removed periodically from the reaction system and placed in a boiling water bath for 15 min to inactivate the enzyme, then they were centrifuged to separate oil and water phases, and the oil phases were dried using anhydrous Na_2SO_4. A sample of 0.1 g was taken from each oil phase (containing TG, DG, MG, and FFA), dissolved in 10 mL chloroform, and subjected to Iatroscan TH-10 TLC/FID analyzer. The relative amounts of TG, 1,3-DG, 1,2-DG, 2-MG, 1-MG, and FFA were determined as described under Analysis of Anchovy Oil.

Fractionation of Reaction Mixture and Analysis of its Fractions

Separation and recovery of the tri-, di-, and monoglyceride fractions from the reaction mixture were carried out by column chromatography on Florisil *(25)*. A glass column (30 × 1.9 cm id) was packed with 25 g of Florisil (100–200 mesh), deactivated with water (7%), and slurried in hexane. Approximately 1 g of sample was dissolved in 10 mL of hexane and applied evenly to the top of the column. Development with 200 mL hexane:diethyl ether (85:15, v/v) eluted TG; DG and MG were eluted with 250 mL hexane:diethyl ether (50:50, v/v), and 200 mL diethyl ether, respectively. Solvent flow was adjusted to 2 mL/min, and the eluates were collected in 10-mL fractions. The separation was monitored by TLC, using silica gel G (Merck) plates, development with hexane:diethyl ether:acetic acid (70:30:1, v/v/v), and visualization with iodine vapor *(26)*. The pure glyceride fractions (TG, DG, and MG) were obtained after pooling of pure fractions and evaporation of solvent.

For the removal of FFA from the reaction mixture, another sample of reaction mixture (2 g) was dissolved in hexane (50 mL), and titrated with aqueous 0.5 *N* NaOH. The aqueous phase was then neutralized with 0.5 *N* HCl. FFA were extracted with diethyl ether and recovered by evaporation of solvent.

Fatty acid composition of each TG, DG, MG, and FFA fraction was determined by capillary GC, as described under Analysis of Anchovy Oil.

Determination of Optimal Reaction Conditions

Several sets of hydrolysis reactions, as described previously, were carried out for 5 h to optimize the extent of hydrolysis at varying process vari-

ables. pH values of 3.0–8.0, temperatures between 30 and 45°C, and the enzyme concentration from 300 to 700 U/g oil were studied.

After establishing the optimal pH, temperature, and the enzyme concentration, in view of determining the optimal reaction time, another set of experiments was run at these optimal conditions prolonging the heating period to 18 h. These experimental results are given in Table 4 and Fig. 6.

All data in tables and figures are averages of triplicate runs.

RESULTS AND DISCUSSION

The main oil characteristics and the fatty acid composition of anchovy oil are given in Tables 1 and 2. Of the 22 fatty acids found in Turkish anchovy oil, 16:0 (19.4%), 16:1 (9.0%), 18:1 (22.0%), 20:5 (EPA) (7.6%), and 22:6 (DHA) (12.7%) fatty acids were the major components. The saturated fatty acid content of anchovy oil was 31.4%. The mono-, di-, tri-, and polyunsaturated fatty acids accounted for 34.0, 5.5, 2.0, and 27.1%, respectively. Turkish anchovy oil contained 7.6% EPA and 12.7% DHA, the two polyunsaturated fatty acids considered to be of major importance in terms of human health. Even though no scientific study was found explaining the positional distribution of PUFA on the glycerol backbone of TG from anchovy oil, it can be deduced that the distribution of these PUFA might follow the same pattern as previously reported for many fish oils *(27,28)*, with location preferentially in the 2-position of the glycerides surrounded by saturated and monounsaturated acids.

At first, to see the effect of pH on the course of hydrolysis of anchovy oil by *R. miehei* lipase, a set of experiments was conducted at large interval's of pH. The extent of hydrolysis was followed by the change in concentration of TG in the reaction mixture. As seen in Fig. 1, *R. miehei* lipase exhibited particularly high activity at pH 4.0. This enzyme was not suitable for alkaline hydrolysis, and it was almost inactive at pH 3.0. This may be explained by the structural change occurring in enzyme proteins caused by pH variation. It has been previously mentioned that for most enzymes the variation of activity with pH, within a range of two or three pH units each side of the isoelectric points, is normally a reversible process. Extremes of pH will, however, cause essentially irreversible denaturation. In alkaline solutions (pH > 8.0), there may be partial destruction of cystine residues caused by base-catalyzed β-elimination reaction; in acid solutions (pH < 4.0), hydrolysis of the labile peptide bonds sometimes found next to aspartic acid residues may occur *(29)*. Similarly, these reactions might have occurred in *R. miehei* enzyme proteins, causing low activity at these pH values.

The optimum pH for the hydrolysis of anchovy oil was established as 4.0. Experiments were conducted at pH 4.0 throughout this study, and the pH did not change over the course of the reaction.

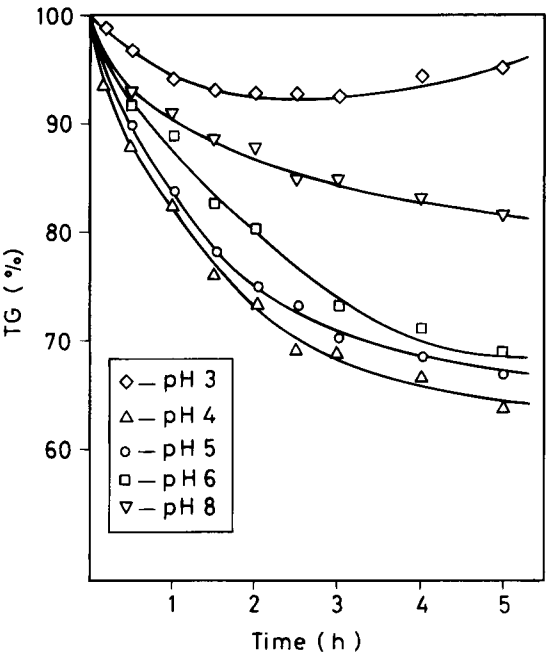

Fig. 1. The effect of pH on the extent of hydrolysis at 40°C. (The amount of enzyme used was 300 U/g oil. The ratio of oil to buffer was 1:1 [v/v].)

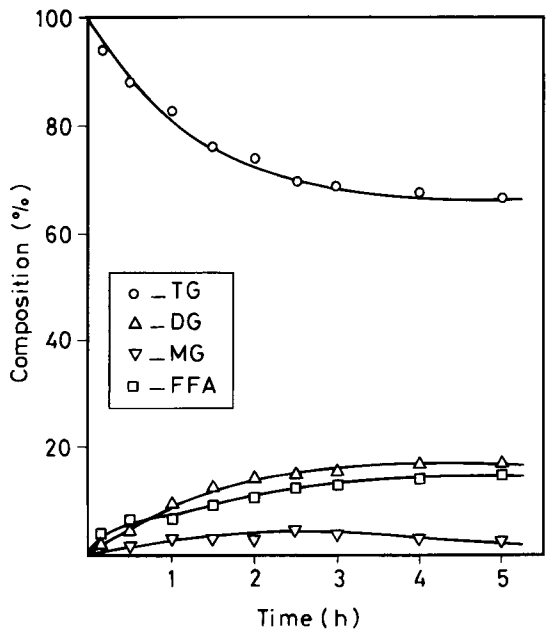

Fig. 2. Change in composition of the reaction mixture with time at pH 4.0 and 40°C. (The amount of enzyme used was 300 U/g oil. The ratio of oil to buffer was 1:1 [v/v].)

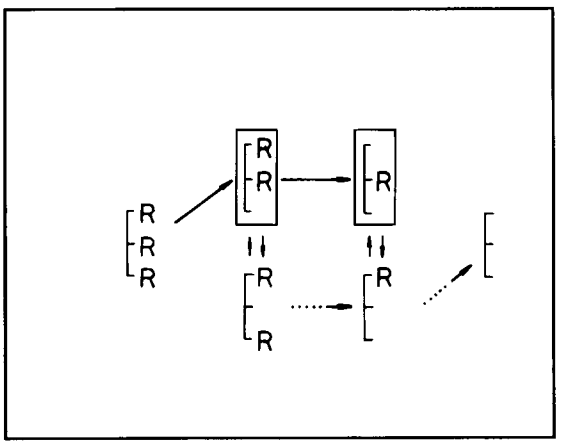

Fig. 3. Possible reaction sequence in glyceride hydrolysis by 1,3-specific lipase enzyme *(30)*. E, Glycerol; R, acyl group; □, product accumulated by the enzymatic reaction; →, hydrolytic reaction; ···≫, hydrolytic reaction detected only after prolonged reaction; and ↑↓, spontaneous isomerization.

The experimentally observed changes in the concentration of TG, DG, MG, and FFA in the reaction mixture during the hydrolysis of anchovy oil at 40°C are shown in Fig. 2. The shapes of all curves revealed that anchovy oil was not hydrolyzed according to the expected reaction mechanism shown in Fig. 3 *(30)*. In general, the enzymatic hydrolysis of TG with a 1,3-specific lipase takes place in a regioselective manner as follows: A 1,3-specific lipase regiospecifically hydrolyzes ester bonds in the 1- and 3-positions, leading to 1,3-specific products, first a mixture of the 1,2- and 2,3-DG, and eventually the 2-MG and the corresponding fatty acids.

In the presence of moisture, acid, and silica gel *(31)*, the isomerization of 1 *(3)*, 2-DG to 1,3-DG, and the conversion of 2-MG to 1-MG, the opposite reactions also occur, resulting in significant amounts of non-1,3-specific products (1,3-DG and 1-MG) in the reaction mixture. MG as either 2-MG or a mixture of 1-MG and 2-MG, are generally obtained in high yield in 2–4 h of reaction period *(31–32)*. Prolonged reaction time results in a decrease in MG yield, and complete hydrolysis of TG to fatty acids and glycerol will eventually take place.

Previous reports have also shown that the hydrolysis reactions of cocoa butter *(31)* and castor oil *(33)* with 1,3-specific *R. miehei* lipase occurred according to the above-mentioned reaction mechanism, giving MG in yield of 32% after 4 h. Based on these results, the authors had presumed that because PUFA is concentrated in the 2-position of TG, hydrolysis of anchovy oil with 1,3-specific *R. miehei* lipase should produce PUFA-rich MG in the earlier stages of reaction. However, inspection of Fig. 2 also indicates that the reaction mixture was always composed of TG, DG

(1,3-DG), and FFA, with a minimum presence of MG (a mixture of 1-MG and 2-MG); the hydrolysis of TG was incomplete, and, after 3 h, approx two-thirds of TG remained unhydrolyzed in the reaction mixture; 1,3-DG appeared to be the only intermediate, reaching a maximum concentration after 3 h, and was not further hydrolyzed to 1-MG beyond this time. It is very important to point out here that these observed results were dramatically different from those expected, as shown schematically in Fig. 3; it is difficult to explain these results only in terms of 1,3-specificity of this enzyme. Another type of specificity is necessary.

It has recently been reported that *R. miehei* exhibits a strong negative specificity toward PUFA, especially DHA, in the esterification and interesterification reactions of fish oils *(34,35)*. Therefore, it can be deduced that the same lipase has probably possessed similar discrimination against PUFA during the hydrolysis of anchovy oil, thus leading to enrichment of PUFA in the unhydrolyzed TG and DG fractions, which might possibly be attributed to some sort of steric hindrance *(18)*. The detailed fatty acid compositions of the TG, DG, MG, and FFA fractions confirmed that the TG and DG fractions were indeed enriched in PUFA. As is shown in Table 3, as a representative example, the fatty acid data for the reaction mixture after 5 h at 40°C revealed that the level of PUFA was raised from 27.1% in the starting material to 34.4 and 32.4% in the TG and DG fractions, respectively. Particularly, the DHA content increased from 12.7% in the anchovy oil to 16.4% in TG. On the other hand, the FFA fraction contained only a trace of DHA and its PUFA concentration was 1.2%. This again showed that PUFA were poor substrates for this lipase and PUFA containing glycerides were hardly hydrolyzed by this enzyme.

To investigate the effect of temperature on the extent of hydrolysis, a second set of hydrolysis experiments was carried out at 30, 35, 40, and 45°C. As seen in Fig. 4, at 30 and 35°C the hydrolysis proceeded progressively for a period of 5 h. At 40°C, a sharp reduction in the content of TG occurred during the first 2.5 h of reaction, with little change thereafter. This might be related mainly to the thermal stability of the enzyme. Prolonging the heating period might lead to a decrease in the activity of this enzyme at 40°C. *R. miehei* lipase was stable at temperature below 40°C, and it considerably lost its activity above 40°C. The maximum activity was observed at 35°C, and thus 35°C was admitted as optimal temperature for the anchovy oil hydrolysis catalyzed with *R. miehei* lipase.

To see the effect of temperature on the fatty acid composition of the reaction mixture, the fatty acid data for the reaction mixtures obtained at 35°C and 40°C are compared in Table 3. Reducing the temperature from 40 to 35°C resulted in a further decrease in the proportion of unhydrolyzed TG to 59.5%, leading to an increase in the content of PUFA to 37.3%. Concomitantly, a small increase in the DG content occurred with decreasing

Table 3
Major Fatty Acid Components of Original Anchovy Oil and the Reaction Mixtures after 5 h at 35°C and 40°C (pH 4.0; enzyme concentration 300 U/g oil; the ratio of oil to buffer 1:1, v/v).

	Major fatty acids (wt % of total fatty acids)							PUFA in total fatty acids (%)	Enrichment PUFA in total fatty acids (-fold)
	14:0	16:0	16:1	18:1	20:5 (EPA)	22:6 (DHA)	Other PUFAa		
Anchovy Oil	6.5	19.4	9.0	22.0	7.6	12.7	6.8	27.1	1.00
Component of reaction mixture[b] (40°C)									
TG (66.2%)	4.4	16.4	8.1	21.8	9.2	16.4	8.8	34.4	1.27
DG (16.7%)	4.7	13.3	11.5	20.7	9.8	13.3	9.3	32.4	1.20
MG (2.2%)	4.4	17.2	15.2	32.8	6.3	11.9	4.5	22.7	–
FFA (14.9%)	17.4	39.0	10.7	24.6	0.2	<0.1	1.0	1.2	–
Component of reaction mixture[b] (35°C)									
TG (59.5%)	4.0	14.9	7.5	21.3	10.1	17.6	9.6	37.3	1.38
DG (17.4%)	4.7	13.7	11.6	21.6	9.4	12.3	8.9	30.6	1.13
MG (5.3%)	3.4	18.0	16.9	28.6	3.4	7.7	2.3	13.4	–
FFA (17.9%)	16.7	39.8	10.8	25.2	0.3	<0.1	1.2	1.5	–

[a] Includes 16:4, 18:4, 20:4, 22:4, and 22:5 fatty acids.
[b] Wt % of total reaction mixture.

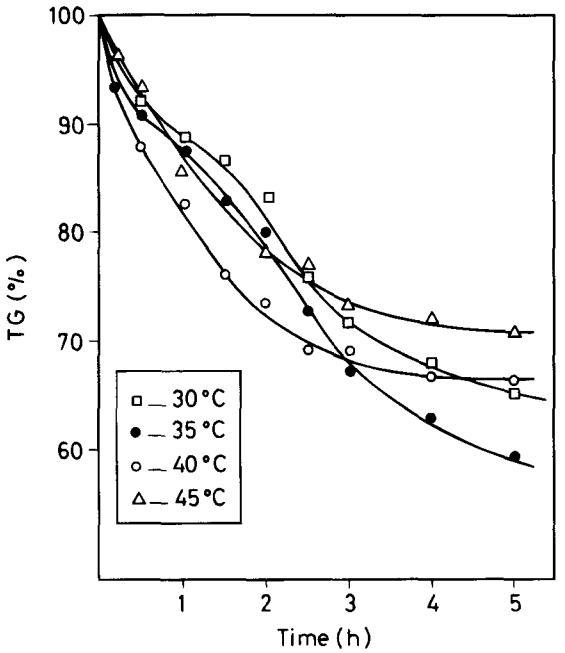

Fig. 4. The effect of temperature on the extent of hydrolysis at pH 4.0. (The amount enzyme used was 300 U/g oil. The ratio of oil to buffer was 1:1 [v/v].)

temperature, causing a substantial reduction in its PUFA content. Almost no release of DHA was observed again in the FFA fraction at 35°C. Most of DHA was concentrated in the TG fraction. A relatively lower concentration of DHA also accumulated in the DG fraction, but not in MG fraction.

After establishing the optimal pH and temperature, to promote hydrolysis as much as possible, and at the same time to increase the content of PUFA in TG and DG fractions, the effect of enzyme concentration on the progression of hydrolysis, and on the fatty acid composition of the resulting reaction mixtures, was also investigated.

As observed in Fig. 5, in the reaction mixture, the TG content decreased sharply with increasing the enzyme concentration in the range of 300–500 U/g oil. At above 500 U/g oil of enzyme concentration, additional increases in concentration caused considerably small changes in the composition of the reaction mixtures within the 3 h reaction period. Moreover, the content of TG gradually increased by prolonging the reaction time to 5 h. Similar behavior has recently been observed for the hydrolysis of coconut oil and lard with lipase from *Aspergillus sp (36).* Therefore, the appropriate amount of enzyme was established as 500 U/g of oil for the hydrolysis of anchovy oil with *R. miehei* lipase.

To determine the optimum reaction time, another set of hydrolysis experiments was run at previously established optimum conditions (pH

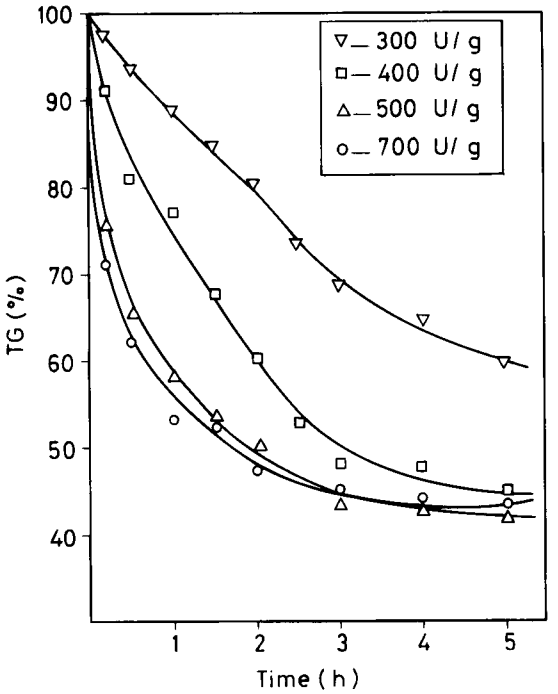

Fig. 5. The effect of enzyme concentration on the extent of hydrolysis at pH 4.0 and 35°C. (The ratio of oil to buffer was 1:1 [v/v].)

4.0, 35°C, and 500 U/g oil of enzyme concentration) for 18 h. As observed in Fig. 6, most of the changes in the composition of the reaction mixtures occurred between 0 time and 3 h. The DG and MG fractions reached to maximum values 26.9 and 7.4%, respectively, and approx 44% of TG remained unhydrolyzed in the reaction mixture after 3 h. Prolonging the reaction time to 18 h resulted in considerably smaller decreases in the TG, DG, and MG contents of the reaction mixture.

In Table 4, the fatty acid compositions of the reaction mixtures after 3 and 18 h are compared. After 3 h of reaction, with this lipase, the level of PUFA was raised from approx 27% in the original anchovy oil to approx 45 and 30% in the TG and DG fractions, respectively. No enrichment of PUFA was observed in the MG fraction. On the other hand, the FFA fraction contained only 1.8% PUFA, corresponding to 1.6% of total PUFA in the original oil.

Extending the reaction time from 3 to 18 h slightly increased the level of PUFA in the TG and DG fractions, namely by 1.5 and 2.8%, respectively; however, it caused a loss of large amounts of PUFA (about 7% of total PUFA) to the FFA fraction. After 18 h, the final PUFA content in the MG fraction was 20.9%, which was still less than in the original anchovy oil.

According to our experimental results, it was noticed that the PUFA content both in the glyceride (TG, DG, and MG) and FFA fractions

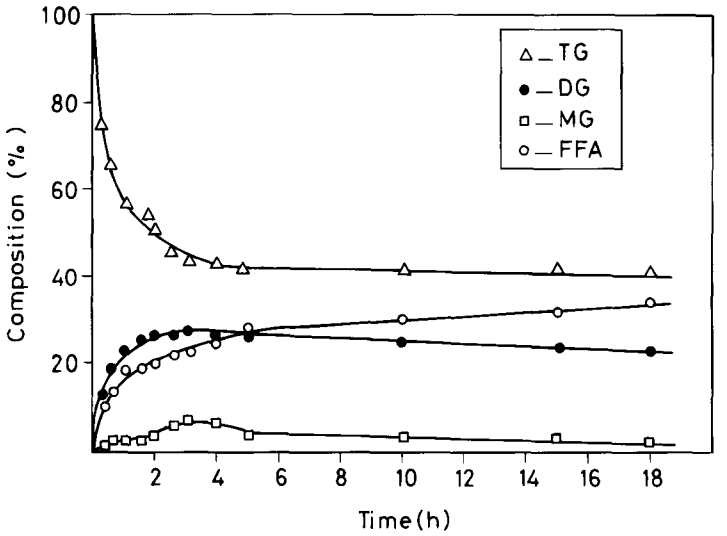

Fig. 6. Change in composition of the reaction mixture with time at pH 4.0 and 35°C. (The amount enzyme used was 500 U/g oil. The ratio of oil to buffer was 1:1 [v/v].)

increased as hydrolysis progressed. In order to promote hydrolysis as much as possible, and at the same time cause minimum PUFA loss, the final appropriate reaction time was admitted as 3 h.

Finally, anchovy oil was hydrolyzed with *R. miehei* lipase at optimum conditions (pH 4.0, 35°C, 3 h, and 500 U/g oil of enzyme concentration). FFA in the reaction mixture were removed by titration and the glyceride mixture was obtained, consisting of 56.0% TG, 34.5% DG, and 9.5% MG. The PUFA content in the glyceride mixture was found to be 39.6%, which was 1.7 times more than that in the original anchovy oil. This glyceride mixture contained mostly PUFA-rich TG (PUFA content, 45.3%) and DG (29.6%), but not MG. Particularly, DHA was concentrated in TG (23.0%) among three glyceride fractions. The above results indicated that *R. miehei* lipase was indeed resistant to PUFA, especially to DHA, during the hydrolysis process. It released PUFA extremely slowly, resulting in their accumulation in the TG and DG fractions, particularly in TG. In addition to fatty acid specificity, *R. miehei* seems to recognize the whole TG structure, in other words, to have a triglyceride specificity, which has been defined by Tanaka et al. *(18)*, in the hydrolysis of tuna oil with *C. cylindracea*, as a comparative difference of activity to TG species. Probably, the saturated and monoenoic acids in TG not containing PUFA were more easily hydrolyzed with this lipase than those in TG containing PUFA. As a result, the TG levels in the glyceride mixtures stayed high and the unhydrolyzed TGs were rich in PUFA, especially in DHA.

In conclusion, the data presented here show that *R. miehei* lipase could be used for the concentration of PUFA in specific glyceride fractions, especially in TG during the hydrolysis of anchovy oil, and a glyceride mixture

Table 4
Major Fatty Acid Components of Original Anchovy Oil and the Reaction Mixtures after 3 and 18 h reaction times at 35°C (pH 4.0; enzyme concentration 500 U/g oil; the ratio of oil to buffer 1:1, v/v).

	Major fatty acids (wt % of total fatty acids)							PUFA in total fatty acids (%)	Enrichment PUFA in total fatty acids (-fold)
	14:0	16:0	16:1	18:1	20:5 (EPA)	22:6 (DHA)	Other PUFA[a]		
Anchovy oil	6.5	19.4	9.0	22.0	7.6	12.7	6.8	27.1	1.00
Component of reaction mixture[b] (3 h reaction time)									
TG (43.7%)	3.7	12.4	6.1	17.6	11.5	23.0	10.8	45.3	1.67
DG (26.9%)	5.0	14.4	10.0	23.1	9.9	10.0	9.7	29.6	1.09
MG (7.4%)	4.3	18.0	15.0	29.1	3.7	6.4	2.1	12.2	–
FFA (22.0%)	13.5	37.3	12.3	28.6	0.4	0.1	1.3	1.8	–
Component of reaction mixture[b] (18 h reaction time)									
TG (40.4%)	3.2	12.1	5.9	17.6	11.7	25.1	10.0	46.8	1.73
DG (23.1%)	4.9	14.5	10.7	26.2	11.2	12.0	9.2	32.4	1.20
MG (2.6%)	1.2	15.7	13.4	27.9	5.8	12.2	2.9	20.9	–
FFA (33.9%)	11.5	31.1	12.0	25.3	1.8	0.4	4.3	6.5	–

[a] Includes 16:4, 18:4, 20:4, 22:4, and 22:5 fatty acids.
[b] Wt % of total reaction mixture.

enriched in PUFA with up to 40% could be obtained from anchovy oil of Turkish origin, with losing only about 2.0% of PUFA in the FFA fraction.

ACKNOWLEDGMENT

This work was supported by Istanbul Technical University Research Foundation. We thank Novo-Nordisk A/S, Denmark, for the donation of *R. miehei* lipase.

REFERENCES

1. Dyerberg, J. and Bang, H. O. (1979), *Lancet*, 433–435.
2. Kristensen, S. D., Schmidt, E. B., Andersen, H. R., and Dyerberg, J. (1988), *Artery*, **15,** 316–329.
3. Weaver, B. J. and Holub, B. J. (1988), *Prog. Food Nutr. Sci.* **12,** 111–150.
4. Darlington, L. G. (1988), *Ann. Rheumatis Dis.* **47,** 169–172.
5. Ackman, R. G. (1992), *Fatty Acids in Foods and Their Health Implications*, Chow, C. K., ed., Marcel Dekker, New York, pp. 169–184.
6. Revankar, G. D. (1974), *J. Food Sci. Technol.* **11,** 27–28.
7. Tuelsner, M. (1990), *Lebensmittel-industrie*, **37,** 271–273.
8. Roggero, J. P. and Coen, S. U. (1981), *J. Liquid Chromatogr.* **4,** 1817–1829.
9. Halgunset, J., Lund, E. W., and Sunde, A. (1982), *J. Chromatogr.* **237,** 496–499.
10. Adolf, R. O., and Emken, E. A. (1985), *J. Am. Oil Chem. Soc.* **62,** 1592–1595.
11. Abe, Y. and Tanaka, M. (1984), *Fette Seifen Anstrichmittel*, **86,** 8–10.
12. Arai, M., Fukuda, H., and Morikawa, H. (1987), *J. Ferment. Technol.* **65,** 569–574.
13. Mishra, V. K., Temelli, F., and Ooraikul, B. (1983), *Food Res. Int.* **26,** 217–226.
14. Nilsson, W. B., Gauglitz, E. J., and Hudson, J. K. (1989), *J. Am. Oil Chem. Soc.* **66,** 1596–1600.
15. El Boustani, S., Collette, C., Monnier, L., Descomps, B., Crastes de Paulet, A., and Mendey, F. (1987), *Lipids* **22,** 711–714.
16. Haraldsson, G. G., Hoskuldsson, P. A., Sigurdsson, S. T., Thorsteinsson, F., and Gudbjarnason, S. (1989), *Tetrahedron Lett.* **30,** 1671–1674.
17. Tanaka, Y., Hirano, J., and Funada, T. (1992), *J. Am. Oil Chem. Soc.* **69,** 1210–1214.
18. Tanaka, Y., Funada, T., Hirano, J., and Hishizume, R. (1993), *J. Am. Oil Chem. Soc.* **70,** 1031–1034.
19. Hoshino, T., Yamane, T., and Shimizu, S. (1990), *Agric. Biol. Chem.* **54,** 1459–1467.
20. Osada, K. and Hatano, M. (1991), *J. Jpn. Oil Chem. Soc.* **40,** 1037–1040.
21. Adachi, S., Okumura, K., Ota, Y., and Mankura, M. (1993), *J. Fermentation and Bioeng.* **75,** 259–264.
22. Maehr, H., Zenchoff, G., and Coffen, D. L. (1994), *J. Am. Oil Chem. Soc.* **71,** 463–467.
23. Ustün, G., Akova, A., and Dandik, L. (1996), *J. Am. Oil. Chem. Soc.* **73,** 389–391.
24. Dandik, L., Arioğlu, G., and Aksoy, H. A. (1993), *Appl. Biochem. Biotechnol.* **42,** 119–126.
25. Litchfield, C. (1972), *Analysis of Triglycerides*, Academic, New York, pp. 19–20.
26. Mangold, H. K. (1965), in *Thin-Layer Chromatography*, Stahl, E., ed., Springer-Verlag, Heidelberg, pp. 137–181.
27. Hamilton, R. J. and Rossell, J. B. (1986), *Analysis of Oils and Fats*, Elsevier, London and New York, p. 332.
28. Aursand, M., Jorgensen, L., and Grasdalen, H. (1995), *J. Am. Oil Chem. Soc.* **72,** 293–297.
29. Chaplin, M. F. and Bucke, C. (1990), *Enzyme Technology*, Cambridge University Press, Cambridge, p. 16.

30. Borgström, B. and Brockman, H. L. (1984), *Lipases*, Elsevier, Amsterdam, New York, p. 458.
31. Goh, S. H., Yeong, S. K., and Wang, C. W. (1993), *J. Am. Oil Chem. Soc.* **70,** 567–570.
32. Holmberg, K. and Österberg, E. (1990), *Progr. Colloid Polym. Sci.* **82,** 181–189.
33. Ghoshray, S. and Bhattacharya, D. K. (1992), *J. Am. Oil Chem. Soc.* **69,** 85–88.
34. Langholz, P., Andersen, P., Forskov, T., and Schmidtsdorff, W. (1989), *J. Am. Oil Chem. Soc.* **66,** 1120–1123.
35. Pedersen, S. B. and Holmer, G. (1995), *J. Am. Oil Chem. Soc.* **72,** 239–243.
36. Fu, X., Zhu, X., Gao, K., and Duan, J. (1995), *J. Am. Oil Chem. Soc.* **72,** 527–531.