Production of Structured Lipids in a Packed-Bed Reactor with *Thermomyces lanuginosa* Lipase

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ABSTRACT: Lipase-catalyzed interesterification between fish oil and medium-chain TAG has been investigated in a packed-bed reactor with a commercially immobilized enzyme. The enzyme, a *Thermomyces lanuginosa* lipase immobilized on silica by granulation (Lipozyme TL IM; Novozymes A/S, Bagsvaerd, Denmark), has recently been developed for fat modification. This study focuses on the new characteristics of the lipase in a packed-bed reactor when applied to interesterification of TAG. The degree of reaction was strongly related to the flow rate (residence time) and temperature, whereas formation of hydrolysis by-products (DAG and FFA) were only slightly affected by reaction conditions. The degree of reaction reached equilibrium at 30–40 min residence time, and the most suitable temperature was 60°C or higher with respect to the maximal degree of reaction. The lipase was stable in a 2-wk continuous operation without adjustment of water content or activity of the column and the substrate mixture.

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KEY WORDS: Fish oil, interesterification, Lipozyme TL IM, packed-bed reactor, structured lipids, *Thermomyces lanuginosa* lipase.

A number of interesting products from lipase-catalyzed modification of oils and fats have been reported, such as cocoa butter-like fats, human milk fat substitutes, chocolate antibloom agents, partial acylglycerols, modified fish oil products, margarine fats, structured lipids, and other related lipid products (1,2). Structured lipids have attracted particular attention recently owing to their potential applications in nutrition (3,4).

Lipases have been widely accepted as biocatalysts for the modification of oils and fats (5,6). One of the concerns for future commercial and industrial exploitation of lipases remains the cost, which is still the main factor involved in new process development and the economical outcome of industrial-scale production. This concern is especially true for the common commodity oils and fats or products therefrom, which are commercialized at a relatively low price.

A recently developed silica-granulated *Thermomyces lanuginosa* lipase, named Lipozyme TL IM (Novozymes A/S, Bagsvaerd, Denmark), has been claimed to have a significantly low price (7). This immobilized lipase has been commercialized to target the production of commodity oils and fats such as margarine fats. Our previous study showed that the lipase had high interesterification activity comparable to the previously developed Lipozyme RM IM (*Rhizomucor miehei;* Novozymes A/S) lipase in batch reactors (8). In both 1-kg-scale experiments and 300-kg pilot plant test productions, the lipase was reused for 10 batches and was stable without any water content readjustment. This was attributed to the highly hydrophilic character of the lipase carrier materials, which can retain sufficient water to maintain the lipase activity. This is obviously an advantage in the continuous operation of packed-bed reactors.

In this study, the operation in the continuous packed-bed reactor was carried out for the production of structured lipids from fish oil and medium-chain TAG (MCT). The basic parameters of flow rate and temperature and the lipase stability were studied to demonstrate the promising aspects of this new commercial lipase for further development of its applicability.

MATERIALS AND METHODS

Materials. Refined fish oil was provided by Aarhus Olie (Aarhus, Denmark). MCT containing 60 mol% caprylic acid (8:0) and 40 mol% capric acid (10:0), was from Grünau GmbH (Illertissen, Germany). The FA compositions of the fish oil, its blend with MCT, and the *sn*-2 position TAG are given in Table 1. Lipozyme TL IM was from Novozymes A/S; it is an *sn*-1,3-specific lipase from *T. lanuginosa* with silica granulation. The water contents of Lipozyme TL IM and the oil blend (fish oil/MCT, 1:1 mol/mol) were 5.8 ± 0.1 and 0.07 ± 0.01%, respectively. All other chemicals and reagents for the analysis were of analytical or chromatographic grade.

Packed-bed reactor and process method. A schematic description of the reactor is depicted in Scheme 1. The operation process, the packing of the column, and the determination of porosity of the bed were as described previously, except that the bed size was changed to 26 mm (i.d.) × 40 cm (length) (9). One enzyme bed was used for all experiments except the final one, for which a new enzyme bed was used from the start. The experiments were run continuously and consecutively without changing the bed between each parameter study. With each new experiment using a specific flow rate or temperature, substrates were pumped into the enzyme bed. The sample was withdrawn after the collection of 300

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TABLE 1
FA Compositions (mol%) and *sn*-2 Position Distributions of Fish Oil, the Blended Mixture[a], the Enzymatically Produced Product (E product)[b], and the Chemically Randomized Product (C-product)[c]

	Fish oil		Blended mixture			E-product			C-product		
FA	Total	*sn*-2	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3
8:0	—	—	29.5	30.5	29.0	29.0	29.8	28.6	29.3	27.5	30.2
10:0	—	—	20.3	19.2	20.9	20.7	19.6	21.3	20.5	20.9	20.3
14:0	8.8	12.1	4.3	6.3	3.3	4.2	6.1	3.3	4.2	4.5	4.1
15:0	0.7	1.3	0.3	0.5	0.2	0.3	0.5	0.2	0.3	0.3	0.3
16:0	18.6	24.6	9.2	12.1	7.8	9.4	12.4	7.9	9.0	9.8	8.6
16:1	7.1	7.5	3.5	3.6	3.5	3.3	3.3	3.3	3.4	3.4	3.4
17:0	0.7	0.4	0.3	0.1	0.4	0.3	—	0.5	0.3	0.3	0.3
17:1	0.6	0.8	0.3	0.4	0.3	0.3	0.4	0.3	0.3	0.4	0.3
18:0	2.4	0.6	1.1	0.2	1.6	1.3	0.2	1.9	1.5	1.1	1.7
18:1n-9	10.1	4.5	4.8	2.3	6.1	4.9	2.5	6.1	4.9	5.5	4.6
18:1n-7	2.2	0.9	1.2	0.5	1.6	1.0	0.4	1.3	1.1	1.3	1.0
18:2n-6	1.9	1.6	1.0	0.8	1.1	1.0	0.9	1.1	1.0	1.0	1.0
18:3n-3	1.5	1.7	0.6	0.8	0.5	0.5	0.7	0.4	0.6	0.6	0.6
18:4	4.7	4.6	2.5	2.4	2.6	2.7	2.2	3.0	2.4	2.2	2.5
19:0	0.8	1.0	0.3	0.4	0.3	0.3	0.4	0.3	0.3	0.3	0.3
20:1n-9	6.6	1.7	3.3	0.8	4.6	3.2	0.8	4.4	3.3	3.0	3.5
20:5n-3	10.3	12.4	5.5	6.3	5.1	5.6	6.5	5.2	5.6	5.7	5.6
22:1n-9	10.2	2.0	4.8	0.7	6.9	4.9	0.8	7.0	4.9	4.9	4.9
22:5n-3	0.7	1.6	0.3	0.9	0.0	0.4	0.9	0.2	0.3	0.3	0.3
22:6n-3	12.0	20.2	6.0	11.1	3.5	6.1	11.3	3.5	6.2	5.8	6.4
Σ	99.9	99.7	99.1	99.9	98.7	99.4	99.7	99.3	99.4	98.9	99.7

[a]Fish oil (Aarhus Olie, Aarhus, Denmark) and medium-chain TAG (Grünau GmbH, Illertissen, Germany), 1:1 mol/mol.
[b]Lipozyme TL IM-catalyzed interesterified fish oil (Lipozyme TL IM) and medium-chain TAG were supplied by Novozymes A/S (Bagsvaerd, Denmark).
[c]See the Materials and Methods section for details.

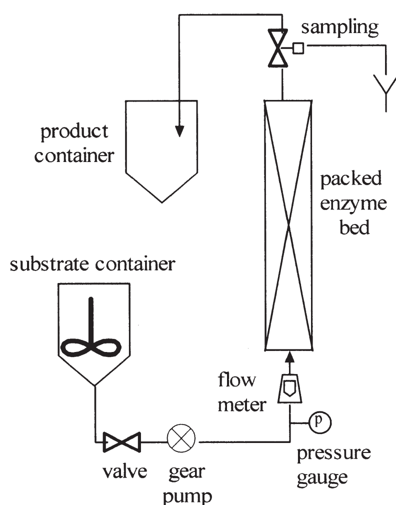
mL of product, equivalent to three enzyme bed void volumes. Following sampling, the equipment was adjusted to the new experimental conditions (flow rate or temperature) for the following experiments. To test stability, the conditions were fixed and samples were taken regularly during the running of the experiments. In all operations, substrates were protected with N_2 gas.

Chemical randomization. Randomization was conducted in a conventional 45-L pilot refining vessel. The stirring was done by an impeller stirrer. Temperature was controlled by a jacket with steam. Vacuum and N_2 gas were connected in order to remove air from the reaction system. The blended oil mixture (fish oil/MCT 1:1, mol/mol) was loaded into the reactor, and stirring was regulated to 230 rpm. The temperature was increased to 90°C after vacuum reached about 100 mbar. The temperature was maintained at 90°C for 30 min to dry the oil. Temperature was then reduced to 60°C and 0.1 wt% sodium methoxide (based on the oil mixture) was added under stirring. The air in the reactor was removed by N_2 and vacuum. The reaction was stopped after 30 min by addition of a citric acid water solution. The oil was washed 3–4 times until the pH was below 7. The oil was dried again under vacuum for 30 min at 90°C. The mixture was then cooled to room temperature before being transferred to containers.

TAG profile analysis by HPLC. TAG profiles were analyzed by RP-HPLC as described previously (10). A high-performance liquid chromatograph (JASCO Corporation, Tokyo, Japan) was used with two pumps, a solvent-mixing module, an autosampler, and an evaporative light-scattering detector (ELSD; Sedere, Alfortville, France). The separation was performed on a Supelcosil LC-18 column (length = 25 cm, i.d. = 4.6 mm, particle size = 5 µm; Supelco, Bellefonte, PA) with a binary solvent system of acetonitrile (solvent A) and isopropanol/hexane (solvent B: 2:1, vol/vol). A linear gradient of solvent B from 35 to 65 over 60 min was applied at a flow rate of 1 mL/min, followed by 100% of solvent B for 6 min. The lipid samples were dissolved in chloroform (50 mg/mL), and 10 µL was injected. The TAG were expressed in terms of the relative percentages of the total TAG after normalization. Duplicate determinations were made. The relative SD for peaks above 1% were less than 5%. The change in the amount of tricaprylin before and after reaction was used to monitor the degree of reaction, as no other TAG have the same ECN (equivalent carbon number) value of 24. Both DHA and EPA have the same ECN of 10 as capric acid. Therefore, all other peaks containing the three FA (EPA, DHA, and capric acid) were complicated to interpret. The chromatograms of the mixture before and after reaction are given in Figure 1. The tricaprylin peak was further identified by MS as described below. Therefore, the degree of interesterification reaction was defined as

$$\text{degree of reaction} = \frac{P_0 - P_E}{P_0 - P_R} \quad [1]$$

where P_0 is the area percentage of tricaprylin peak of the blend before reaction, P_E is the area percentage of tricaprylin peak of enzymatically produced products, and P_R is the area percentage of tricaprylin peak of the chemically randomized product.



SCHEME 1

DAG content analysis by HPLC. DAG in the products were analyzed by HPLC on a Hewlett-Packard narrow-bore silica column (length = 10 cm, i.d. = 2.1 mm, particle size = 5 μm; Hewlett-Packard, Waldbronn, Germany) as described previously (11). DAG content was calculated based on total sample amount. The concentration of DAG was expressed as the weight percentage (wt%) of the product samples.

Peak identification by MS. An HP 1100 Series LC/MSD system (Hewlett-Packard) fitted with an atmospheric pressure chemical ionization source was used for peak identification of TAG as described previously (12). The ionization source was used in positive mode and the solvent vapors reacted as the reagent gas. The corona voltage was 3000 V, the vaporizer temperature was 400°C, and the nebulizer gas pressure was 60 psi. The heated nitrogen drying gas temperature was 325°C with a flow rate of 4.0 L/min. The same column and solvents as described in the TAG HPLC section were used. Fifty millimoles ammonium acetate was used as postcolumn addition at a flow rate of 0.1 mL/min. System control and data evaluation were conducted using HP ChemStation (Hewlett-Packard).

Grignard degradation. The *sn*-2 position FA profile was determined following Grignard degradation as described previously (8).

GC analysis of FAME. Oils and *sn*-2 monoacylglycerols (MAG) were methylated by the methanolic potassium hydroxide method (13). The diethyl ether in the extract was first evaporated with N_2. The *sn*-2 MAG were then redissolved in 0.3 mL heptane and methylated with 30 μL 2 M KOH methanol solution. After drying and centrifuging, the methyl esters were analyzed on a gas chromatograph equipped with an FID as described previously (9). Three determinations were conducted and the average was used. The SD for all peaks were less than ±0.2 mol%.

FFA content in products. FFA contents (%) were determined by AOCS official methods (13). An average M.W. of 221.6 of the blend was used for calculation.

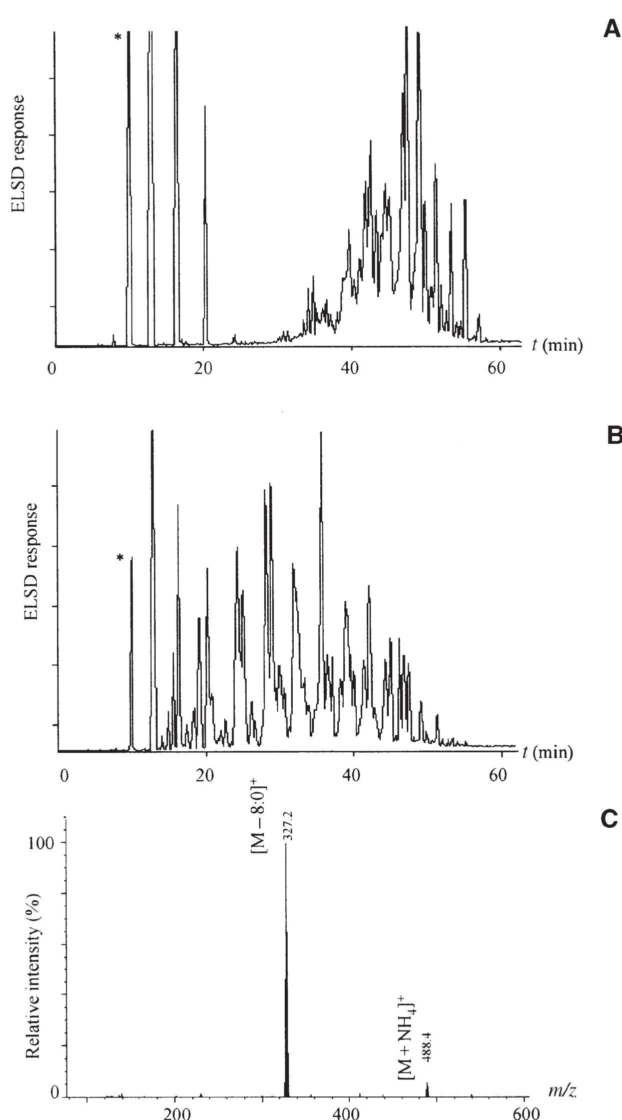


FIG. 1. Separation of TAG molecular species on a reversed-phase high-performance liquid chromatograph (JASCO Corporation, Tokyo, Japan) with evaporative light-scattering detection (ELSD). (A) Chromatogram of the mixture of fish oil and medium-chain TAG (MCT) before reaction, and (B) the chromatogram of the mixture after reaction. The peaks marked with an asterisk represent the tricaprylin peak; (C), atmospheric pressure chemical ionization mass spectra of the tricaprylin peak. The ion *m/z* 327.2 represents the DAG fragment ion $[M - 8:0]^+$, whereas the ion *m/z* 488.4 represents the ammonium adduct molecular ion $[M + NH_4]^+$.

Water content in oils and lipases. Water contents were analyzed by the Karl Fischer titration method. Triplicate measurements were made.

RESULTS AND DISCUSSION

The reaction between fish oil (LLL, long-chain TAG where L = long chain length) and MCT (MMM, medium-chain TAG where M = medium chain length) either by Lipozyme TL IM-catalyzed interesterification or chemical randomization will produce a

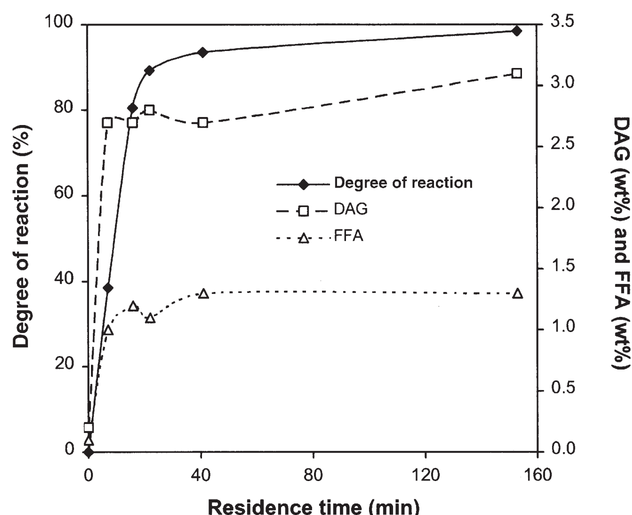


FIG. 2. Effect of residence time (flow rate) on the degree of reaction, and DAG and FFA contents of the Lipozyme TL IM (Novozymes A/S, Bagsvaerd, Denmark) catalyzed interesterification between fish oil and MCT. Reaction conditions: temperature 60°C and no water addition. See Figure 1 for abbreviation.

variety of different TAG such as LLM, LML, LMM, MLM, and so on, not to mention different FA in both LLL and MMM. As a consequence, the content of MMM (or LLL), which is 50% at the start, will be reduced after reaction. In choosing tricaprylin from MMM as a special indicator in which the peak is not complicated by any other TAG, the mixture of the starting material contains 7.4% tricaprylin and the product contains 2.2% after chemical randomization. Therefore, the reaction degrees can be calculated using Equation 1.

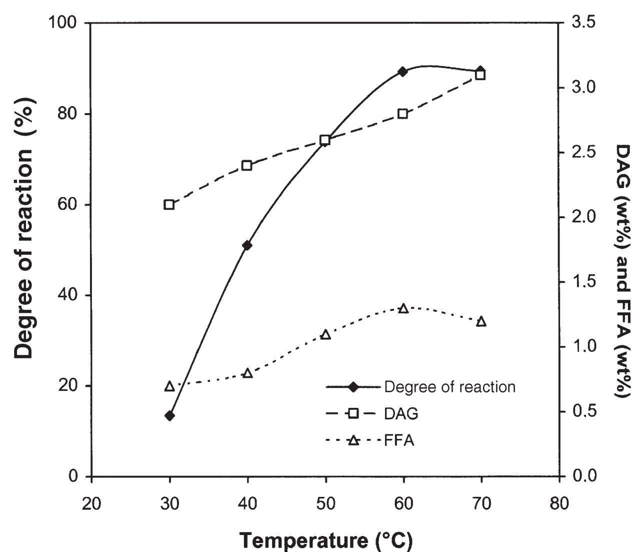


FIG. 3. Effect of temperature on the degree of reaction, and DAG and FFA contents of the Lipozyme TL IM-catalyzed interesterification between fish oil and MCT. Reaction conditions: residence time 22 min and no water addition. See Figure 2 for supplier of Lipozyme TL IM and Figure 1 for abbreviation.

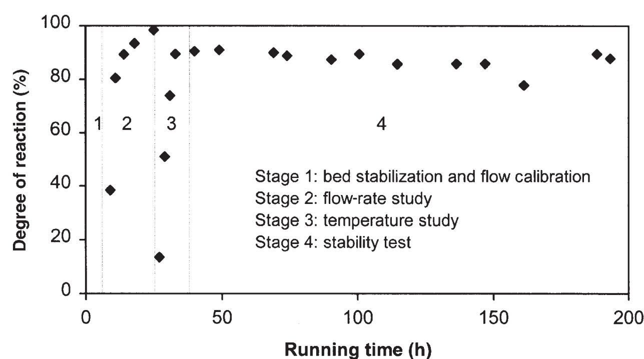


FIG. 4. Stability test in the same column as the parameter study for the Lipozyme TL IM-catalyzed interesterification between fish oil and MCT. Reaction conditions: residence time 40 min, temperature 60°C, and no water addition. See Figure 2 for supplier of Lipozyme TL IM and Figure 1 for abbreviation.

The overall void fraction of the reactor was measured as 0.51 ± 0.1 using a previously developed method (9). Therefore, residence time was calculated according to the following equation:

$$\text{residence time (min)} = V \times \varepsilon / V_f \qquad [2]$$

where V is the enzyme bed volume (mL), ε is the void fraction, and V_f is the volume flow rate (mL/min). Based on this calculation, the time course of the reaction in the packed-bed reactor is described in Figure 2. The curve is similar to that previously observed for acidolysis in packed-bed reactors (9). The concentration of hydrolysis by-products (DAG and FFA) changed little during the operation using different residence times (flow rates). The reason may be that the bed had been equilibrated by feeding substrate for a few hours and that extra water had been removed before the parameter study was initiated.

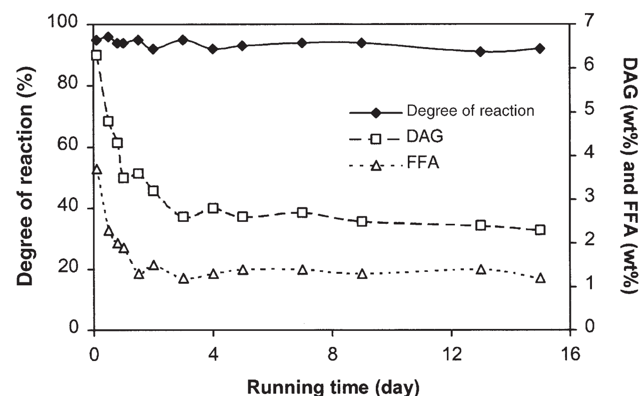


FIG. 5. Stability test in a new packed column with the same dimensions for the Lipozyme TL IM-catalyzed interesterification between fish oil and MCT. Reaction conditions: residence time 45 min, temperature 60°C, and no water addition. See Figure 2 for supplier of Lipozyme TL IM and Figure 1 for abbreviation.

Temperature had a strong effect on the degree of reaction (Fig. 3). This is slightly different from the results in batch reactors in which the degree of reaction was stabilized above 55°C (8). This difference in effect of temperature may result from the use of different substrates, which in the latter experiment had a higher melting point. In both cases, the effect of temperature below 55°C was significantly positive. This might indicate that temperature not only relates to the reactivity of the lipase but also, more importantly, relates to the mass transfer limitations in the granulated lipase. The concentrations of hydrolysis by-products increased only slightly with the increase in temperature (Fig. 3).

The operational stability in the packed bed is one of the important characteristics of biocatalysts for practical applications. From our previous study of batch reactors (8), we expected that the lipase would not lose its activity owing to the loss of water during operation, which is often true for other lipases (9). As seen from Figures 4 and 5, the degree of reaction could be maintained for up to 2 wk in this study without significant reduction. Thus, the lipase was not inactivated by drying owing to continuous substrate flow. The results also imply that the lipase is stable for at least 2 wk. The reason for this effect might be that the carrier has a high hydrophilicity and therefore can bind sufficient water to maintain lipase activity. The stability might also reflect the high load of lipase on the carrier, which lowers the water activity optimum (14). From Figure 5, it can be seen that DAG and FFA were gradually reduced on the first day of operation with the freshly packed bed, indicating that water content was gradually removed until the equilibrium was reached.

Because the lipase is *sn*-1,3-specific, the *sn*-2 positions of the TAG in both MMM and LLL will theoretically not be involved in any acyl exchange. However, acyl migration might occur owing to the formation of DAG as reaction intermediates (15). The results of analyses of FA distributions in different products are given in Table 1. It is obvious that PUFA, especially EPA and DHA, which are mostly located in the *sn*-2 position in the original fish oil, are maintained in the *sn*-2 position in the enzymatically interesterified product compared to the randomized one. This may indicate that the enzymatically produced products possess benefits compared to the chemically produced ones because the location of EPA and DHA at the *sn*-2 position improves their absorption (16).

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