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Enzymatic Interesterification of Butterfat with Rapeseed Oil in a Continuous Packed Bed Reactor

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Lipase-catalyzed interesterification of butterfat blended with rapeseed oil (70/30, w/w) was investigated both in batch and in continuous reactions. Six commercially available immobilized lipases were screened in batch experiments, and the lipases, Lipozyme TL IM and Lipozyme RM IM, were chosen for further studies in a continuous packed bed reactor. TL IM gave a fast reaction and had almost reached equilibrium with a residence time of 30 min, whereas RM IM required 60 min. The effect of reaction temperature was more pronounced for RM IM. TL IM showed little effect on the interesterification degree when the temperature was raised from 60 °C to 90 °C, whereas RM IM had a positive effect when the temperature was increased from 40 °C to 80 °C. Even though TL IM is an *sn*-1,3 specific lipase, small changes in the *sn*-2 position of the triacylglycerol could be seen. The tendency was toward a reduction of the saturated fatty acid C14:0 and C16:0 and an increase of the long-chain saturated and unsaturated fatty acids (C18:0 and C18:1), especially at longer residence times (90 min). In prolonged continuous operation the activity of TL IM was high for the first 5 days, whereafter it dramatically decreased over the next 10 days to an activity level of 40%. In general, the study shows no significant difference for butterfat interesterification in terms of enzyme behavior from normal vegetable oils and fats even though it contains short-chain fatty acids and cholesterol. However, the release of short-chain fatty acids from enzymatic reactions makes the sensory quality unacceptable for direct edible applications.

KEYWORDS: Interesterification; interesterification degree; lipases; Lipozyme TL IM; Lipozyme RM IM; packed bed reactor; butterfat; butter oil; milkfat

INTRODUCTION

During recent years a shift in consumer preference is seen toward products containing less and healthier fat (*1*). Since the 1990s the consumption of butter and ghee has been decreasing in the developed countries (*2*). This change has given rise to new challenges for the dairy industry to develop new uses for the surplus of butterfat. Butterfat possesses some favorable properties both physically and organoleptically. For this reason butterfat is used in bread, cakes, cookies, and ice creams where it gives good structure and texture and imparts a buttery flavor and aroma as well as a creamy mouthfeel. Butterfat also has some good nutritional qualities as a source of essential fatty acids and fat-soluble vitamins, but due to the high content of saturated fatty acids such as lauric, myristic, and palmitic acid residues it has been claimed to be hypercholesterolemic (*3*, *4*). This tendency to cause a raised blood cholesterol level can potentially lead to coronary heart disease (CHD) and other related diseases (*4*−*6*). However, the cause of CHD is multifactorial, and dietary saturated fats are not the only reason. The results of studies on the etiology of heart disease are inconclusive and sometimes contradictory (*7*). For this reason the focus in development of new butter products should be on improvements of the poor melting profile of butter, and to a lesser extent an effort to reduce the amount of saturated fatty acids. By simple blending of butterfat with vegetable oils the melting profile and the fatty acid composition of the butter product can be changed, making it more spreadable, the nutritional properties are improved, and the desirable organoleptic attributes are maintained (*8*). Another way to overcome the problems associated with butterfat is modification by chemical or enzymatic interesterification. By chemical interesterification of butterfat with vegetable oil a randomization of the fatty acid residues on the glycerol backbone of the triacylglycerols takes place, and a product with a desired melting profile and improved nutritional properties can be obtained. Both chemical and enzymatic interesterification can give rise to off-flavors due to enzymatic release of short-chain fatty acids in enzymatic interesterification and due to oxidation and processing at higher temperature in

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Table 1. Basic Characteristics of the Oils and Fats Including Fatty Acid Composition (FAC) (mol %), Peroxide Value (PV), Water Content, and Free Fatty Acid (FFA) Content

fatty acids and others	anhydrous butterfat	rapeseed oil	sunflower oil	fully hydrogenated cottonseed oil
C4:0	10.8			
C6:0	4.2			
C8:0	2.0			
C10:0	3.6			
C12:0	3.8			
C14:0	11.2			1.9
C16:0	27.5	5.3	6.8	33.8
C18:0	9.1	1.6	3.3	57.5
C18:1n-9	20.0	60.7	25.9	5.2
C18:2n-6	1.4	20.1	62.7	1.0
C18:3n-3	0.8	10.2	0.4	0.3
C20:0			0.2	0.3
others	5.5	2.1	0.7	
PV (mequiv/kg)	0.03	0.02	0.31	0.00
water (%)	0.12	0.05	0.07	0.05
FFA (%)	0.32	0.07	0.11	0.15

chemical interesterification (*9*). Removal of off-flavors by deodorization has to be done.

Enzymatic interesterification with lipase catalysis has certain advantages over chemical catalysis such as milder reaction conditions and regiospecificity (*10*, *11*), which may be less harmful to the butter flavor (*12*).

So far there have been only a limited number of studies on enzymatic modification of butterfat, and most of the emphasis has been on the performance on the laboratory scale without much consideration of product properties and process technology for industrial scales (*13*−*15*). This study is part of ongoing research that has the purpose to develop a new process, which can be adapted by the industry, to modify butterfat through enzymatic interesterification with rapeseed oil. Rapeseed oil has been widely used for the manufacture of butter as a blend to increase the nutritional values of butter as well as to improve the butter spreadability. The oil contains a large amount of oleic acid and a good mixture of linoleic (ω6) and linolenic (ω3) acids as shown in **Table 1**. The process includes further refining to remove the off-flavors formed during the interesterification in subsequent study. The modified product should have a better melting profile and higher nutritional value, while maintaining the attractive texture, flavor, and aroma of butterfat. Compared to simple blending, the new product should have an extended shelf life. The objective of this study is to select the best commercially available lipase and find the optimal conditions for a continuous enzymatic interesterification process.

MATERIALS AND METHODS

Materials. Anhydrous butterfat was a generous gift from Arla Foods (Holstebro, Denmark). Rapeseed oil, fully hydrogenated cottonseed oil, and sunflower oil were obtained from Aarhus United (Aarhus, Denmark), Bunge Oil Inc. (White Plains, NY), and Karlshamns AB (Karlshamn, Sweden), respectively. The basic characteristics of the oils and fats are listed in **Table 1**. Lipozyme TL IM (batch no. Lab 5000103), Lipozyme RM IM, and Novozym 435 were all obtained from Novozymes A/S (Bagsvaerd, Denmark). Lipase AK-20 and Lipase PS-D-I were obtained from Amano Enzyme Inc. (Nagoya, Japan). Other characteristics of the lipases are given in **Table 2**. Cholesterol (95−98%) and butyric acid (>99%) were purchased from Sigma-Aldrich (St. Louis, MO). Tributyrin (98%) was obtained from Riedel-de Haën (Hanover, Germany). All other reagents and solvents were of analytical grades.

Enzymatic Interesterification. For the batch reactions, 20 g of a mixture of anhydrous butterfat/rapeseed oil (70/30, w/w) was added to a 50 mL conical flask and placed in a water bath at 50 °C with magnetic stirring at 400 rpm. The reaction was initiated by adding 10 wt % enzyme, and samples were withdrawn from the system every 2 h of the 10 h reaction. No water was added.

For the continuous reactions, a packed bed reactor (length, 20 cm; inner diameter, 1.5 cm), equipped with a heating jacket connected to a water bath for temperature control and fully packed with immobilized enzyme, was used. The desired amounts of the mixture of anhydrous butterfat and rapeseed oil (70/30, w/w) were pumped into the reactor at a specified volumetric flow rate via a small pump from FMI Fluid Metering (Syosset, NY). From the outlet of the reactor the interesterified product could be collected or a sample could be withdrawn. To prevent solidification of the oil mixture the whole setup, excluding the water bath, is kept in a small Plexiglas room heated to 60 °C. To obtain the desired residence time (Rt) the volumetric flow rate (V_f) can be set to a value calculated from the following formula:

$$\mathrm{Rt} = V\frac{\epsilon}{V_f}$$

where ϵ is the porosity of the immobilized enzyme and V is the volume of the reactor.

Analysis of Triacylglycerol Composition by HPLC. The triacylglycerol (TAG) composition was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) based on the equivalent carbon number (ECN). ECN is defined as CN − 2n, where CN is the number of carbons in the TAG (excluding the three in the glycerol backbone) and n is the number of double bonds. The HPLC system was a JASCO high-performance liquid chromatograph (Jasco Corporation, Tokyo, Japan) equipped with two PU-980 pumps, an HG-980-30 solvent mixing module, an AS-950 autosampler, a UV-970 UV/vis detector, and a Sedex 55 evaporative light-scattering detector (ELSD, Sedere, Alfortville, France). The ELSD was operated at a temperature of 40 °C with air as the nebulizing gas at 2.2 bar. The column used was Supelcosil LC-18 (250 × 4.6 mm, Supelcosil Inc., Bellefonte, PA) packed with a particle size of 5 μm. The mobile phase was a binary

Table 2. The Immobilized Commercial Lipases Used and Their Characteristics

brand	lipase species	carrier	specificity	water content (wt %)	porosity
Lipozyme TL IM	*Thermomyces lanuginosus*	silica granules	*sn*-1,3-specific	6.0	0.77
TL-lab-immobilized	*Thermomyces lanuginosus*	Accurel EP 100	*sn*-1,3-specific	5.8	
Lipozyme RM IM	*Rhizomucor miehei*	macroporous resin	*sn*-1,3-specific	3.2	0.45
Novozym 435	*Candida antarctica* lipase B	macroporous polymer based on methyl and butyl methacrylic esters	nonspecific	4.1	0.65
Lipase PS-C-I	*Burkholderia cepacia*	ceramic particles	nonspecific[a]	4.3	
Lipase PS-D-I	*Burkholderia cepacia*	diatomaceous earth	nonspecific[a]	3.7	

[a] No clear claim.

solvent system of acetonitrile (solvent A) and 2-propanol/hexane (2:1, v/v) (solvent B). The applied flow rate was 1 mL/min, and a gradient program was employed, consisting of a linear gradient of solvent B from 25% to 45% over 60 min, going to 100% over 2 min and holding for 6 min, after which it decreases to 25% over 2 min and holds for 5 min. The samples were dissolved in chloroform (10 mg/mL), and 10 μL was injected. Triacylglycerol peaks were identified by TAG standards from their ECN. Three determinations were conducted for all the analyses, and the average was used for result evaluation. The selected peaks of ECN48 and ECN36 had a relative standard deviation below 3.7% and 3.3%, respectively.

Determination of Cholesterol. The content of cholesterol in the samples was determined as described by Fletouris *et al*. (*16*). The samples (0.2 g) are saponified in capped tubes with 0.5 M methanolic KOH solution by heating for 15 min at 80 °C. Water is added to the mixtures, and the unsaponifiable fractions are extracted with hexane before further analysis by capillary GC. The gas chromatography system consisted of a HP 6890 series chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a flame-ionization detector (FID) and a capillary column (Zebron ZB-5, 60 m × 0.25 mm, 0.2 μm film thickness; Phenomenex Inc., Torrance, CA). Helium was used as carrier gas with a column flow rate of 1.3 mL/min. The injector was used in split mode with a ratio of 1:21. The oven temperature was increased from 280 °C to 310 °C over 20 min. The injector and the detector temperature were 320 °C and 310 °C, respectively. The cholesterol was quantified using stigmastanol as internal standard. Three determinations were conducted, and the average was used for result interpretation. The relative standard deviation was below 6.1%.

Fatty Acid Composition. Fatty acid methyl esters (FAME) were prepared from triacylglycerols through transesterification catalyzed by KOH in methanol (*17*). The FAME dissolved in heptane was analyzed by gas−liquid chromatography (GLC) with a HP 6890 series chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a flame-ionization detector (FID) and a fused-silica capillary column (Supelco SP2380, 60 m × 0.25 mm, i.d., 0.25 mm film thickness; Supelco Inc., Bellefonte, PA). The carrier gas was helium with a column flow rate of 1.2 mL/min. The injector was used in split mode with a ratio of 1:20. Oven temperature was programmed from 50 (3 min) to 160 °C at a rate of 15 °C/min, increased to 182 °C at a rate of 1 °C/min, further to 200 °C at a rate of 10 °C/min, and finally to 225 °C at a rate of 30 °C/min and held for 12 min. The injector and detector temperatures were 260 °C and 300 °C, respectively. The fatty acid methyl esters were identified by comparison of their retention times with standards from Sigma Chemical (St. Louis, MO). Three determinations were conducted for all analyses. The averages were used for result evaluation. The relative standard deviations were varied a bit larger when conducting in different days in the repeatability test where the maximum was observed as 8.2%. For samples from the same set of experiments, analysis was conducted in the same running of the instrument. In such cases, the relative standard deviations were much lower with none higher than 2.7%.

***sn*-2 Fatty Acid Composition.** Fatty acid composition at the *sn*-2 position was determined by Grignard degradation with allylmagnesium bromide followed by isolation and methylation (*18*). The *sn*-2 MAG fraction was isolated by thin-layer chromatography on boric acid impregnated thin-layer chromatography plates developed twice (2 × 60 min) in chloroform/acetone (90:10, v/v), methylated and analyzed by gas−liquid chromatography as described above. All samples were conducted for three determinations including GC analysis for fatty acid composition. The average was used for result interpretation. The relative standard deviations were below 4.2% for all the samples from the same set of experiments.

Determination of Free Fatty Acids. One gram of oil is weighed (4 decimals) and dissolved in 20 mL of chloroform. Then 25 mL of ethanol and 5 drops of phenolphthalein indicator are added, and the mixture is titrated with a 0.1 M sodium hydroxide solution until the appearance of a pink color. The content of FFA is calculated as palmitic acid as

$$\%\ \mathrm{FFA} = \frac{(\mathrm{mL_a} - \mathrm{mL_b})m(256.4 \times 100)}{1000g}$$

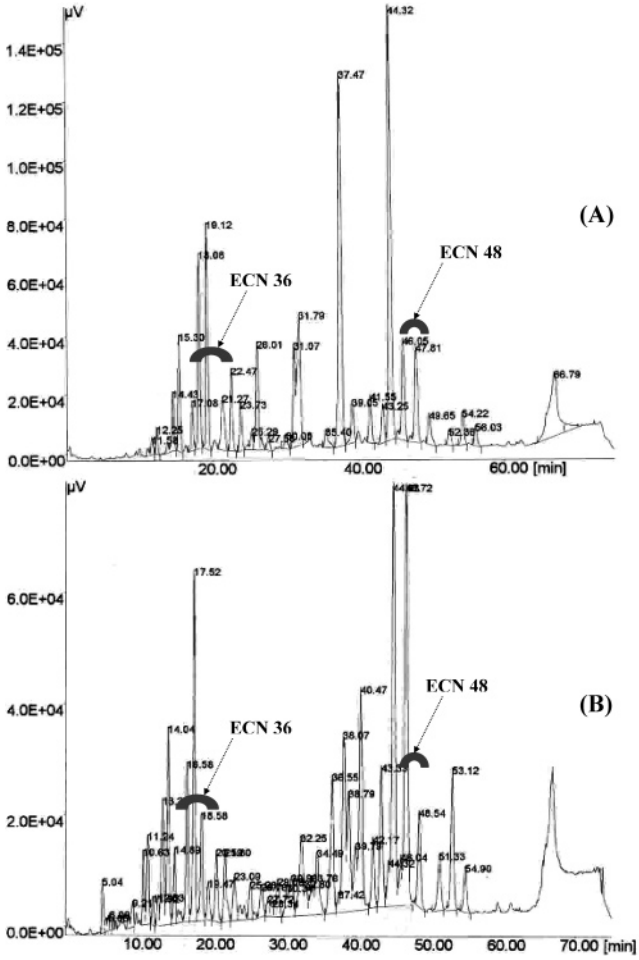

Figure 1. HPLC chromatograms showing the two groups of peaks used for calculation of the interesterification degree. (**A**) HPLC chromatogram of a blend consisting of butterfat and rapeseed oil (70/30, w/w). (**B**) HPLC chromatogram of the same blend after enzymatic interesterification in a continuous packed bed reactor. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); enzyme, Lipozyme TL IM; enzyme amount, approximately 15 g; reaction temperature, 60 °C; room temperature, 60 °C; flow rate, 0.9 mL/min; residence time, 30 min.

where $\mathrm{mL_a}$ is the amount of sodium hydroxide, $\mathrm{mL_b}$ is the amount of sodium hydroxide used on a blind sample, *m* is the molarity of sodium hydroxide, 256.4 is the molar mass of palmitic acid, and *g* is the amount of oil. Double determinations were conducted, and the average was used for result evaluation. The method was very repeatable, and the differences between the double determinations were below 0.04% for all the samples.

Evaluation Method of Interesterification Extent. The activity of the lipase, as indicated by the extent of rearrangement of the fatty acids in the triacylglycerols, is calculated on the basis of the change in two peak groups in the HPLC chromatograms for the triacylglycerol composition (*11*). For a blend of butterfat and rapeseed oil (70/30, w/w) a great change in peak areas after reaction is seen in a group of peaks of ECN48, whereas the peak areas of a group of peaks of ECN36 are relatively stable during the reaction progress (**Figure 1**). The interesterification degree (ID) is therefore defined as:

$$\mathrm{ID} = \frac{\text{peak area (\%) of ECN48}}{\text{peak area (\%) of ECN36}}$$

In the evaluation of effects of butyric acid and cholesterol on the lipase behavior, butterfat cannot be used since it contains these two compounds. In such cases, the blends of sunflower oil and fully hydrogenated cottonseed oil (80/20, w/w) were used. Different peaks appeared from the butterfat and rapeseed oil mixture as shown in **Figure 1**.

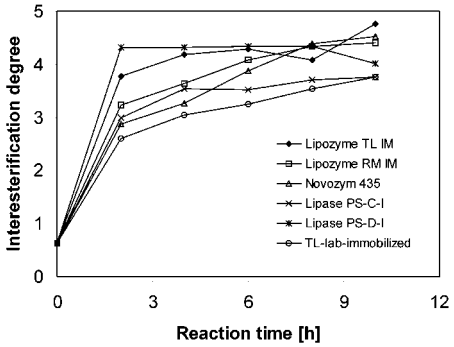

Figure 2. Comparison of the interesterification degree of six different lipases in batch interesterification of a butterfat and rapeseed oil blend (70/30, w/w). The enzyme amount was 10% (w/w); the reaction temperature was kept at 50 °C and magnetic stirring at 400 rpm. No addition of water.

Through evaluation of a time course of the reaction with these two oils, we selected two groups of peaks as well in which one group had a big change during the reaction and the other group had little change during the reaction progress. Therefore, the interesterification degree was defined as the ratio of the peak areas between the two groups.

Sensory Evaluation. An expert panel, consisting of three trained persons, performed preliminary sensory evaluations of the butter products. The oil samples were thawed in a water bath for 4 h, followed by heating at 50 °C for 30 to 60 min before the sensory evaluation. The evaluation was done by smelling and tasting the samples, and a scale for total off-flavor from 0 to 10 was used, where 0 is given to oils with no off-flavor and 10 is given to oils with an extremely high off-flavor. The panel agreed on a score for each product. Therefore, no statistics could be made on the data.

RESULTS AND DISCUSSION

Lipase Screening. For butterfat interesterification with plant oils using enzyme approaches, few studies considered the availability of commercial immobilized lipases in terms of possibility of industrial implementation. In this study 6 immobilized lipases were used and 5 of them are available from commercial sources. **Figure 2** shows a comparison of six different commercially available immobilized lipases in batch reactions. The performance of the lipase is evaluated on the basis of the interesterification degree calculated from specific peak areas of the HPLC chromatograms as described in the Materials and Methods section. A preliminary study has shown the method to be reproducible with a CV below 5% for four identical experiments (results not shown). All six lipases were capable of catalyzing the interesterification between butterfat and rapeseed oil (70/30, w/w), but Lipase PS-D-I, Lipozyme TL IM, and Lipozyme RM IM seem to give a faster reaction with a higher final interesterification degree than the other enzymes. For Lipase PS-D-I the highest interesterification degree is seen already after 2 h of reaction, and the level is maintained during the total reaction time of 10 h, indicating that equilibrium has been reached. For Lipozyme TL IM the highest interesterification degree is seen after 6 h of reaction, whereas the interesterification degree of the other lipases is gradually increasing during the 10 h of reaction. Differences in the formation of free fatty acids are also seen between the different lipases. Lipase PS-D-I has the lowest formation of FFA at 2.8%, followed by Novozym 435 at 3.6%, Lipozyme RM IM at 4.0%, Lipase AK 20 at 5.3%, Lipozyme TL IM at 6.0%, and TL lab-immobilized with the highest FFA formation at 9.4%. The amount of free fatty acid formed is associated with the water content in the reaction system and the water bound in the enzyme granules of the immobilized lipases. An increasing level of water leads to a higher degree of hydrolysis. For an *sn*-1,3 specific lipase-catalyzed interesterification, FFA and *sn*-1,2 (2,3)-DAG are formed as byproducts by hydrolysis. **Table 3** shows the fatty acid composition in the *sn*-2 position of a butterfat and rapeseed oil blend after 10 h of reaction with the six different lipases. Compared to the oil blend, small changes are observed in the *sn*-2 position in all cases, even though only three lipases are nonspecific (Novozym 435, Lipase PS-D-I, and Lipase PS-C-I) and therefore catalyze the interesterification in all three positions on the glycerol backbone. The reason for this can be acyl migration, where the chemically unstable *sn*-1,2 (2,3)-DAG undergoes acyl migration to produce 1,3-DAG. One of the acyls in the 1,3-DAG can migrate to the *sn*-2 position to form an *sn*-1,2 (2,3)-DAG, and now the 1,3-specific lipase can catalyze the re-esterification yielding a TAG where the acyl in the *sn*-2 position has changed (*19*). Acyl migration has been shown to be dependent on reaction time, water content, enzyme dosage, and types of enzyme carrier (*20*). The process should be optimized to minimize the acyl migration. The two lipases, Lipozyme TL IM and Lipozyme RM IM, were used for further investigations in continuous interesterification reactions, taking account of the activity, specificity, and availability in large quantity. These two lipases were not used for such modification of butterfat in previous studies.

Parameter Study. Figure 3 shows the interesterification degree as a function of the residence time in a packed bed reactor for the continuous interesterification catalyzed by Lipozyme TL IM and Lipozyme RM IM. For a residence time under 60 min Lipozyme TL IM gives a faster reaction than Lipozyme RM IM, but at residence times above 60 min the two enzymes give comparable results. Above a residence time of 30 min, Lipozyme

Table 3. *sn*-2 Position Fatty Acid Composition (mol %) of a butterfat and Rapeseed Oil Blend (70/30, w/w) after 10 h of Batch Interesterification at 50 °C and with Magnetic Stirring at 400 rpm

fatty acids	mixed oil	Lipozyme TL IM	Lipozyme RM IM	Novozym 435	Lipase PS-C-I	Lipase PS-D-I	TL-lab-immobilized
C10:0	2.32	2.29	2.91	2.58	3.09	2.20	2.15
C12:0	3.57	2.91	3.49	3.05	3.19	2.71	3.00
C14:0	13.39	9.35	12.15	10.19	9.62	8.23	10.76
C14:1	0.80	0.71	0.73	0.72	0.67	0.74	0.80
C16:0	28.95	26.96	28.07	26.68	25.39	25.11	26.25
C16:1	1.56	1.26	1.43	1.35	1.22	1.05	1.28
C18:0	3.65	6.94	4.42	5.71	6.34	7.37	4.61
C18:1n-9	23.87	29.50	24.95	27.64	27.64	27.84	23.42
C18:1n-7	0.50	1.15	0.66	0.93	1.11	1.23	0.68
C18:2n-6	10.40	8.03	9.65	9.61	7.49	6.02	8.28
C18:3n-3	4.30	3.25	3.96	3.87	3.03	2.24	2.99
others	6.69	7.65	7.58	7.67	11.21	15.26	15.78

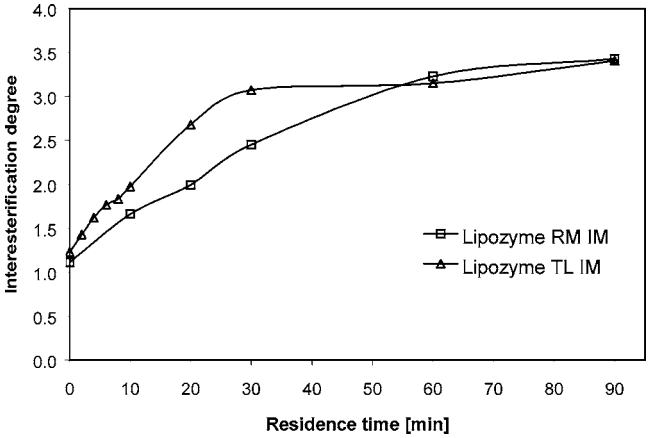

Figure 3. Effect of residence time on the interesterification degree in a continuous reaction. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, butterfat and rapeseed oil (70/30, w/w); enzymes, Lipozyme TL IM and Lipozyme RM IM; enzyme amount, approximately 15 g in both cases; reaction temperature, 60 °C; room temperature, 60 °C.

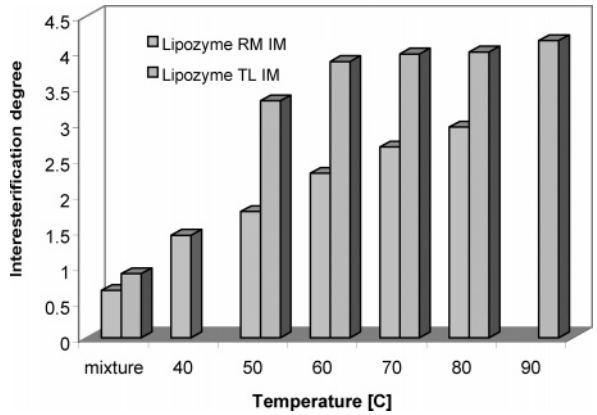

Figure 4. Effect of temperature on the interesterification degree in a continuous reaction. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, butterfat and rapeseed oil (70/30, w/w); enzymes, Lipozyme TL IM and Lipozyme RM IM; enzyme amount, approximately 15 g in both cases; flow rate, 0.9 mL/min; residence time, 30 min; room temperature, 60 °C.

TL IM shows little improvements in interesterification degree, and this time is therefore chosen for the further studies. For Lipozyme RM IM the temperature has a strong effect on the degree of reaction as shown in **Figure 4**. The interesterification degree gradually increases when the reaction temperature is changed from 40 °C to 80 °C. These results differ from those of Lipozyme TL IM, where the temperature has a positive effect on the reaction between 50 °C to 60 °C, after which the interesterification degree is more or less stable from 60 °C to 90 °C. Overall the interesterification degree seen for Lipozyme TL IM is higher than for Lipozyme RM IM. This is partly due to the residence time used in both experimental series. A residence time of 30 min was used in both cases, which favors the faster reacting Lipozyme TL IM. If a residence time of 60 min had been chosen instead, the same effects of the temperature would have been seen, but the difference in interesterification degree between the two enzymes would have been smaller. The observation of these two lipases in butterfat interesterification is generally in agreement with their uses in margarine modification with tropical plant oils and fats, but certainly some differences also exist (*11*, *21*).

Table 4. Total Fatty Acid Composition and *sn*-2 Positional Distribution (mol %) of a Butterfat and Rapeseed Oil Blend (70/30, w/w) before and after Continuous Interesterification by Lipozyme TL IM in a Packed Bed Reactor[a]

residence time:	0 min		10 min		30 min		90 min	
	total	*sn*-2	total	*sn*-2	total	*sn*-2	total	*sn*-2
C4:0	8.05		6.19		5.95		5.98	
C6:0	3.09	0.19	3.12		3.08		3.07	
C8:0	1.43	1.00	1.53	0.61	1.53	0.41	1.52	0.43
C10:0	2.64	2.88	2.93	2.66	2.92	2.63	2.92	2.61
C12:0	2.76	3.92	2.72	3.62	2.73	3.71	2.74	3.52
C14:0	8.18	14.09	7.87	12.92	7.89	12.70	7.92	11.49
C16:0	21.59	27.01	23.23	27.93	23.28	27.71	23.35	26.90
C16:1	1.36	1.88	1.43	1.94	1.44	1.92	1.44	1.85
C18:0	7.12	4.30	6.93	3.62	6.96	4.18	6.95	5.03
C18:1n-12	1.50	1.28	1.25		1.26		1.26	
C18:1n-9	27.99	25.05	27.85	23.64	27.97	23.99	27.86	25.83
C18:1n-7	1.32	0.56	1.21	0.50	1.22	0.56	1.23	0.75
C18:2n-6	6.45	9.80	7.08	10.62	7.11	10.39	7.09	10.04
C18:3n-3	3.07	4.16	2.94	4.29	2.95	4.26	2.95	4.22
others	3.45	3.88	3.72	7.65	3.71	7.54	3.72	7.33

[a] Experimental conditions: packed bed reactor (200 mm × 1.5 mm) with approximately 15 g of enzyme; reaction temperature 60 °C.

As seen from the batch reaction, changes in the *sn*-2 position are also observed for continuous interesterification in a packed bed reactor. **Table 4** shows the overall and the *sn-2* fatty acid composition for the butterfat and rapeseed oil blend catalyzed by Lipozyme TL IM. There is little change in the overall fatty acid composition for the different residence times, but longer residence times give rise to larger changes in fatty acid composition in the *sn*-2 position due to most likely acyl migration. Having a residence time of 30 min a 5.5%, 9.9%, 2.7%, and 4.2% reduction is seen for lauric, myristic, stearic, and oleic acid, respectively. For palmitic acid a small increase of 2.6% is observed. Saturated fatty acids at the *sn*-2 position are more completely absorbed than when present at the outer positions of the triacylglycerols (*22*). It is commonly stated that the saturated fatty acids C12:0, C14:0, and C16:0 have a detrimental nutritional effect (*4*) and other fatty acids like C18:0 and C18:1n-9 have a "neutral" or positive nutritional effect, respectively (*7*, *23*). If the residence time is increased to 90 min, lauric, myristic, and palmitic acid decrease with 10.2%, 18.4%, and 0.4%, respectively, and stearic and oleic acid increase 17.1% and 3.1%, respectively. So even though the overall changes are small, the outcome of the enzymatic interesterification is a more healthy fat with a reduced content of saturated fatty acids in the *sn*-2 position.

Operational Stability. For industrial applications of a biocatalyst the operational stability in a continuous packed bed reactor is an important issue. **Figure 5** shows the operational stability of Lipozyme TL IM for continuous operation over 30 days. The lipase activity as interpreted by the interesterification degree is not very stable during the operating period. Approximately 4 h after reaction start the interesterification degree is at its highest level, and this activity is more or less maintained until the fifth operating day. The activity then decreases dramatically until day 15, where it reaches a plateau with a relatively low activity level of about 40% of the initial activity. This level is more or less kept for the remainder of the test period. The inactivation of the enzyme seems to be more related to the amount of oil passed through the reactor than to the exposure to high temperatures for prolonged times. In the literature, the stability of the lipase is believed to be associated with oil quality, which in general is characterized by overall

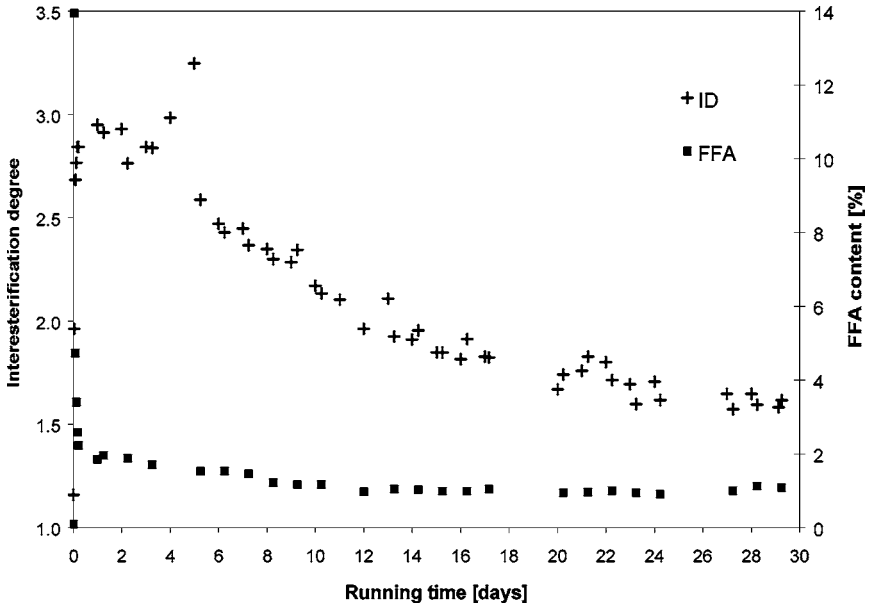

Figure 5. Operational stability test of Lipozyme TL IM in a continuous reaction. Both the interesterification degree and the FFA content are shown. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, butterfat and rapeseed oil (70/30, w/w); enzyme, Lipozyme TL IM; enzyme amount, approximately 15 g; flow rate, 0.9 mL/min; residence time, 30 min; reaction temperature, 60 °C; room temperature, 60 °C.

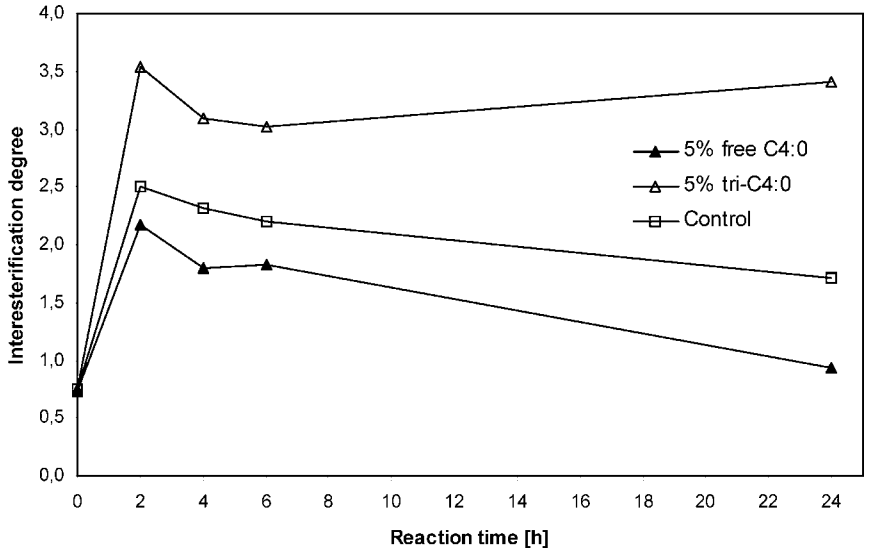

Figure 6. Butyric acid effect on the interesterification degree. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, fully hydrogenated cottonseed oil and sunflower oil (20/80, w/w) + 0% (control), 5% free C4:0, or 5% tri-C4:0, respectively; enzyme, Lipozyme TL IM; enzyme amount, approximately 15 g; flow rate, 0.9 mL/min; residence time, 30 min; reaction temperature, 60 °C; room temperature, 60 °C.

parameters such as primary (hydroperoxides) and secondary (aldehydes and ketones) oxidation products (*24*). In this study, both butterfat and rapeseed oil are not crucial in terms of oxidation since the former contains a large amount of saturated fatty acids and the latter is also regarded stable among vegetable oils. However, butterfat is atypical compared to vegetable oils because it contains butyric acid. This short-chain fatty acid is more hydrophilic than the longer chain fatty acid, and it might impart an effect on the lipase. **Figure 6** shows the interesterification degree of butterfat and rapeseed oil blends with and without 5% butyric acid, in triacylglycerol or in the free fatty acid form. Having butyric acid in its free form seems to have a detrimental effect on the interesterification degree, whereas the opposite effect is observed if butyric acid is added in triacylglycerol form. The latter could possibly just be due to the analysis method used. When tributyrin was introduced to the system, the resulting TAG profiles of the products would be changed and thus the peaks concerned could be affected. Therefore the evaluation method may be the major problem that leads to the increase in interesterification degree. The conclusion on the effect of butyric acid in its free form can be justified though. Short-chain fatty acids are more hydrophilic and lower the pH of the microaqueous layer surrounding the enzyme, which may lead to enzyme deactivation (*25*, *26*). Butyric acid could then potentially lower the activity of the lipase. Different interesterification degrees are therefore caused not only by differences in the behavior of the reaction system. Having butyric acid in its free form might also lead to enzyme deactivation if butyric acid is adsorbed onto the enzyme carrier, blocking the entry of the triacylglycerols and causing the reaction to stop (*27*).

Another compound present in relatively high amount in butterfat is cholesterol, which also can be a factor in the decrease in activity. **Figure 7** shows the interesterification degree of blends of sunflower oil and fully hydrogenated cottonseed oil (80/20, w/w) with different added cholesterol contents. These

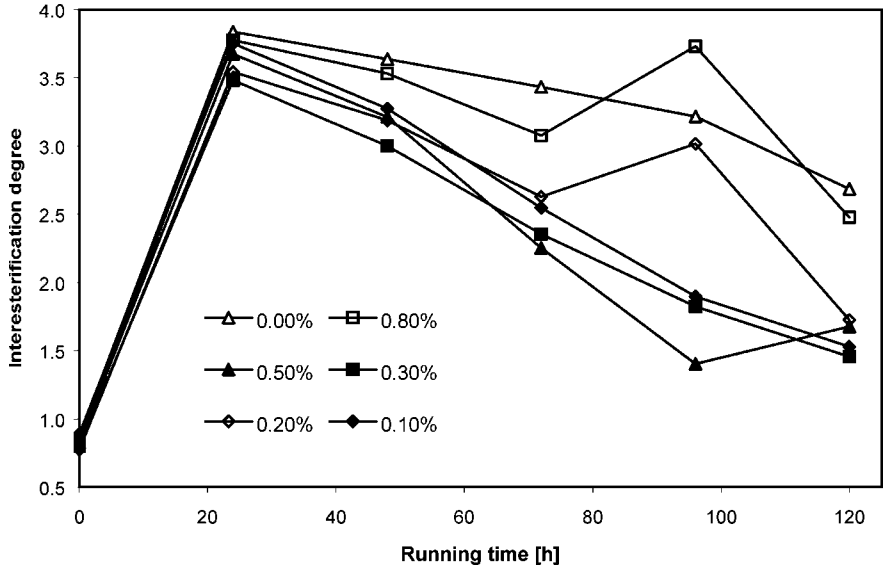

Figure 7. The effect of cholesterol on the interesterification degree. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, fully hydrogenated cottonseed oil and sunflower oil (20/80, w/w) + 0% (control), 0.1%, 0.2%, 0.3%, 0.5%, or 0.8% added cholesterol, respectively; enzyme, Lipozyme TL IM; enzyme amount, approximately 15 g; flow rate, 0.26 mL/min; residence time, 105 min; reaction temperature, 60 °C; room temperature, 60 °C.

oils have very low naturally occurring cholesterol contents. For comparison the blend between butterfat and rapeseed oil (70/30, w/w) has a cholesterol content of 0.24%.

Adding cholesterol to the blend, in general, results in a faster loss in activity compared to the control, even though the results are not statistically significant. It seems that having a moderate level of cholesterol between 0.1% and 0.5% has the most detrimental effect on the lipase, whereas at 0.8% the effect is less pronounced. However, no clear picture between the different levels of cholesterol added can be drawn. The study has been repeated in different operations (results not shown), but this did not shed any new light on the effect of the different levels of cholesterol. Cholesterol is more polar than oils and fats, and it could therefore be hypothesized that the enzyme bed could retain it like a chromatographic column. Some cholesterol may even occupy the surroundings of the lipase particles more closely than the oils and fats. When more and more cholesterol is accumulated in the lipase particles, the polarity situation could be changed, possibly leading to collapse of the cholesterol "layer". A clear elucidation of this special action needs further work. As an overall conclusion from this study, we believe that a significant effect from cholesterol does not exist. A possible small effect of cholesterol will not be an obstacle for the development of the technology in butterfat modification, and this has been confirmed by our large-scale production for longer operation.

The content of FFA is also indicated in **Figure 5**, and one sees a significant high amount in the startup phase, whereafter it fairly quickly reaches a stable and more reasonable level of around 1.0% to 1.5% FFA. Within the first hour of reaction the FFA content peaks at 13.95%, after 2 h it has decreased to 4.71%, and after 4 h it has further decreased to a more acceptable level of 2.59%. The high amount of FFA formed during startup is a consequence of the water content of the immobilized enzyme, and after 4 to 5 h most of the water has been consumed in the high initial degree of hydrolysis. From an industrial application perspective this would mean that the product from the first few hours of startup is waste or at least should be kept separately for a harsher refining. The production can then run without any changes for around 5 days, after which the flow rate has to be lowered to accommodate the loss in enzyme activity. By this way it should be possible to maintain a fairly constant interesterification degree and thereby acquire a more unified product.

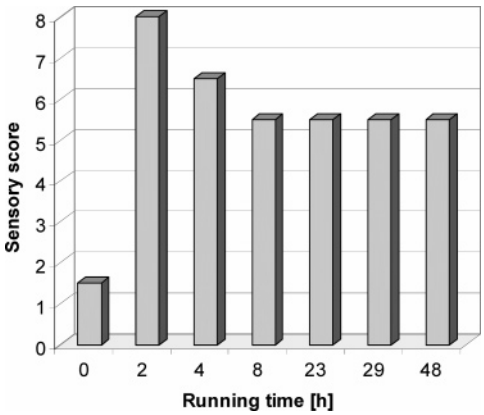

Figure 8. Sensory evaluation of the off-flavor as a function of the running time. Sensory score 0 = no off-flavor, and sensory score 10 = extremely high off-flavor. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, butterfat and rapeseed oil (70/30, w/w); enzyme, Lipozyme TL IM; enzyme amount, approximately 15 g; flow rate, 0.45 mL/min; residence time, 60 min; reaction temperature, 60 °C; room temperature, 60 °C.

A preliminary sensory evaluation of off-flavors indicated that, on a scale from 0 to 10, the interesterified product had an off-flavor at around 5 irrespectively of the reaction temperature and the residence time (results not shown). **Figure 8** shows the sensory evaluation of samples with different running times. It was found that the off-flavor was very high (around 8) after 2 h and decreased some after 4 h, whereafter it reached a constant level from 8 h and forth. This is to be expected, since the amount of FFA is adversely affecting the smell and taste of the butter product. During the first operating hours where the enzyme still contains a lot of water, a high degree of hydrolysis is seen and thus a high amount of FFA is formed. Even though the level of FFA reaches a low constant level after around 5 h, the product is still not acceptable for consumption. In order to obtain a product with an acceptable smell and taste the majority of the

FFA therefore has to be removed by deodorization in a later stage.

Scale-Up of the Reaction System. We have scaled up the reaction system to a larger packed bed reactor (length, 50 cm; inner diameter, 4.7 cm). The volume of the reactor is 867.5 mL, and it holds approximately 400 g of Lipozyme TL IM. Having a residence time of 30 min leads to a daily production of enzymatically interesterified product of close to 30 L. This indicates that by further scale-up it is feasible to use the system in an industrial process, but the interesterified product has to undergo further refining to remove the off-flavor associated with the 1% to 1.5% FFA formed during the reaction. In the long-term continuous reaction, the enzyme activity reduces. If the flow rate is adjusted to accommodate the activity reduction, it should be possible to use the same enzyme for 20 to 30 days.

In general from this study, the packed bed reactor system can be operated for the interesterification of butterfat with plant oils from a small scale to a reasonably large scale. The possible influence of butyric acid and cholesterol in butterfat did not turn out to have a significant impact on the enzyme performance. Lipozyme TL IM has been given particular concern in this study due to its activity and potential low cost in comparison with other lipases used, which was not discussed in detail in above sections. This provides a potential possibility for industrial implementation. The issues of off-flavor production and possible nonspecificity of products due to acyl migration or other reasons should be looked into further.

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