Kilogram-Scale Ester Synthesis of Acyl Donor and Use in Lipase-Catalyzed Interesterifications

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Scaling up of Lipozyme-catalyzed ester synthesis with >99% conversion and a reflux trap to remove product water from the reaction mixture is reported. Ethyl stearate was synthesized in 2000-g batch reactions from technical stearic acid. The ethyl stearate was purified to 97% by crystallization and interesterified with sunflower seed oil by means of a lipase catalyst to investigate reaction parameters of temperature, substrate ratio, enzyme content and catalyst water activity. The endpoint of the reaction was defined as the incorporation of stearate into sunflower seed oil corresponding to the amount of stearate necessary to be incorporated into palm oil mid-fraction to produce a cocoa butter substitute. No tristearate was formed at the reaction endpoint in any of the reactions conducted. Reaction times decreased and levels of free fatty acids and diglycerides increased with increasing temperature and with increasing ratio of acyl donor to triglyceride. Increasing the enzyme content of the reaction mixture reduced reaction times but caused higher levels of free fatty acids and diglycerides. In reactions catalyzed by Lipozyme of defined water activity, the shortest reaction times were obtained at intermediate water activity, while free fatty acid and diglyceride levels increased with water activity. When the interesterification reaction was carried out in refluxing pentane with the condensed solvent dried by passage through a reflux trap, the free fatty acid and diglyceride levels were reduced to 6 and 3.3%, respectively.

KEY WORDS: Diglycerides, ester synthesis, interesterification, Lipozyme, operational stability, reaction temperature, water activity, water removal.

Conducting enzyme-catalyzed reactions in nonaqueous media is presently a well-established discipline and integrates principles and techniques from biochemistry and organic chemistry. These reactions are amenable to large-scale work and have been used for practical applications in several industries. Lipase-catalyzed esterification currently is used to produce a variety of high-purity esters for cosmetic use (1). Lipase-catalyzed resolution of racemic glycidyl butyrate is an important step in industrial production of glycidol of high enantiopurity (2,3). Sunflower seed and safflower seed oils are used to produce cocoa butter substitutes enzymatically in Japan (4).

In our initial investigations into lipase-catalyzed reactions in water-poor media, we attempted the transesterification of ethyl stearate and palm oil mid-fraction to form cocoa butter substitutes (5). Although reaction rates were rapid and a product with the proper incorporation of stearate into only the 1- and 3-positions of the product triglycerides was obtained, the complexity of the palm oil mid-fraction substrate made interpretation of the results for optimization unnecessarily complicated (6). It was difficult to quantitate the diglycerides formed in the reaction mixture, and trisaturated triglycerides were formed due to the presence of saturated fatty acids in the 2-position of the triglycerides of palm oil mid-fraction. This made it impossible to distinguish between trisaturated triglycerides formed as a result of these saturated fatty acids in the 2-position of the substrate and possible trisaturated triglycerides formed by other mechanisms. The mechanisms under consideration were either isomerization of the reaction intermediates promoted by components or parameters of the reaction mixture, or lack of absolute 1,3-specificity of the lipase preparation used. Further investigations were conducted with a model substrate consisting of high-purity triolein and palmitic acid (7). Reaction conditions were elucidated where the formation of trisaturated triglycerides was completely eliminated and diglyceride contents were held as low as possible. Comparison between carboxylic acids and their ethyl esters as acyl donors indicated that the ethyl ester substrates are better substrates due to their faster reaction times and lower production of trisaturated triglycerides. However, high diglyceride contents were obtained in the product when ethyl palmitate was used as the acyl donor. Because ethyl stearate and ethyl palmitate are expensive substrates, a method for their synthesis from free fatty acids was developed (8). In this method, free fatty acids were esterified with a slight molar excess of ethanol in refluxing solvent. The product water generated in ester synthesis was removed from the reaction mixture in the refluxing solvent and was trapped in molecular sieves loaded into a trap. This method proved to be mild enough to synthesize ethyl esters of long-chain polyunsaturated ethyl esters with minimal peroxidation of sensitive double bonds. However, the scale was limited to batches of 50 grams.

In the present report, the scaling up of ester synthesis is reported, with the ester product used for interesterification reactions on a scale of 100 g substrate. Instead of using the highly purified substrates suitable for preliminary investigations, the substrates chosen were of the quality expected to be used in industrial applications. Thus, technical-quality stearic and palmitic acids were used to synthesize ethyl esters in 1- and 2-kilogram batch reactions. The ethyl stearate was triply purified by crystallization and interesterified with sunflower seed oil. The reaction time required to reach an endpoint analogous to the endpoint used in earlier investigations was investigated with the goal of comparing reaction times and by-product formation of acidolysis and interesterification on a small and a larger scale. This endpoint was defined as the incorporation of stearate into sunflower seed oil corresponding to the amount of stearate that must be incorporated into palm oil mid-fraction to achieve a fat with the same content of palmitate, oleate and stearate as cocoa butter. Diglycerides and free fatty acids present in the reaction mixture at this endpoint are reported. In addition, the interesterification reaction was conducted with continuous drying by a reflux trap developed for ester synthesis.

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EXPERIMENTAL PROCEDURES

Sunflower seed oil (Trisun 80), stearic acid and palmitic acid were generous gifts from Karlshamns AB (Karlshamn, Sweden). Lipozyme IM20 (interesterification activity 25 BIU) and Lipozyme IM60 (interesterification activity 66 BIU) were gifts from Novo Nordisk (Bagsvaerd, Denmark). Molecular sieves (3Å) were purchased from Sigma Chemical Co. (St. Louis, MO).

Molecular sieves were activated before use by heating at >250°C overnight. Activated sieves for ester synthesis reactions were pretreated by soaking in absolute ethanol and rinsing with petroleum ether before being exposed to the reaction solvent. Sieves used in interesterification reactions were used after activation only.

Determination of the temperature of refluxing substrate and product mixtures. A three-necked, 250-mL round flask was loaded with substrate (20 g stearic acid plus 5.1 mL ethanol) or product (20 g ethyl stearate). Ten mL solvent (hexane or petroleum ether, 60–70°C) was added through a graduated dropping funnel fitted to one neck of the flask. A thermometer passed through another neck of the flask and was immersed in the liquid. The liquid was heated to reflux, and the temperature was noted after remaining stable for 5 min. Solvent was added through the dropping funnel, and the reflux temperature of the new mixture was determined.

Determination of the dependency of esterification reaction time on Lipozyme content. A reflux trap described previously (8) was filled with hexane and fitted to the reaction vessel. Stearic acid (40–50 g) was dissolved in hexane in the reaction vessel and ethanol (1.25 mol/mol stearic acid) was added. The mixture was heated until vigorous refluxing occurred. Heat was removed until refluxing stopped, and the reaction was started by adding Lipozyme IM60. Samples were withdrawn periodically and the degree of conversion was determined by titration of the free fatty acid substrate remaining. As product water was generated, a water-rich phase separated from the condensed refluxing solvent and fell into the reflux trap. The water-rich phase was removed intermittently and titrated by Karl Fisher coulometric titration to determine the quantity of ethanol by difference. The ethanol lost in the water-rich phase was replaced by adding ethanol to the reaction mixture through a graduated dropping funnel. When the reaction reached about 90% conversion, molecular sieves were added to the drying arm of the reflux trap.

Large-scale ethyl ester synthesis reactions. A 10-L round flask was charged with the acid (500–2000 g), and petroleum ether, 60–70°C (2.5 mL solvent/g substrate fatty acid) was added. Lipozyme IM20 (100 g; 5% by wt of substrate) was added to the reaction mixture and heat was applied. A stirring blade mounted in an overhead motor was operated at 260 rpm. A reflux trap filled with petroleum ether (450 mL) was fitted overhead. After refluxing became vigorous, the reaction was started by adding ethanol (1.25 mol/mol acid substrate) through a dropping funnel. A water-rich phase was collected and ethanol was added as described above. When the water content of the water-rich phase began to decrease, the condensed reflux solvent was diverted through a bed of molecular sieves. The compositions of the technical-quality fatty acids used in this study are given in Table 1.

Determination of initial rates of ester synthesis. Reactions were conducted in round flasks fitted to a reflux trap of either 120 mL (10–300 g syntheses) or 450 mL (300–2000 g syntheses). Initial rates were determined at 30 or 60 min. Reactions were carried out as described above, except that different amounts of solvent were used. In reactions with up to 300 grams of substrate, 3 mL solvent/gram of acid substrate was used and reactions were carried out in round-bottom flasks of up to 2 L. Syntheses of 500 g and above were carried out in a 10-L round-bottom flask. For 500-g syntheses, it was necessary to use 4 mL solvent/gram of acid substrate to ensure that the stirring arm was immersed deep enough for good agitation. For 1-kg and 2-kg syntheses, 2.5 mL solvent/gram of acid substrate was used.

Operational stability of Lipozyme IM20. Reactions were conducted as described above, except that 25 g Lipozyme IM20 was used repeatedly with 1 kg of stearic acid. After each synthesis, the product ester and solvent were removed from the reaction vessel. The Lipozyme remained in the reaction flask, which was recharged with acid for the next synthesis. Solvent was added, and the reaction flask was heated until vigorous refluxing occurred. The reaction was started by the addition of ethanol through a dropping funnel.

Purification of ethyl stearate by crystallization. Ethyl stearate (8 kg) as synthesized above was crystallized at 4°C. A liquid phase formed in the ester mixture, which was enriched in impurities. After 48 h, the liquid was decanted and the ethyl stearate was melted and recrystallized twice.

Interesterification. Lipozyme IM20 with an activity of 25 BIU/g was dried by vacuum to a water activity of 0.07 and used for all experiments. Interesterification reactions were carried out at 50°C with 100 g of substrate,

TABLE 1

Composition of Technical Fatty Acids Used for Ethyl Ester Synthesis and Ethyl Stearate Product After Three Crystallizations (in wt%)

Fatty acid (carbons:double bonds)	Common name	Technical palmitic acid substrate (wt%)	Technical stearic acid substrate (wt%)	Crystallized ethyl stearate product (wt%)
14:0	Myristic acid	2.52	0	0
16:0	Palmitic acid	88.64	8.65	2.5
18:0	Stearic acid	8.32	84.13	96.8
20:0	Arachidic acid	0.52	1.27	0.7
22:0	Behenic acid	0	1.27	0

composed of 2 parts ethyl stearate and 1 part sunflower seed oil by weight, in 350-mL, wide-mouth, screw-capped bottles unless otherwise indicated. Reactions were conducted at temperatures of 30–90°C. Tests of the effects of the substrate ratio were conducted with ethyl stearate/sunflower seed oil in weight proportions of 0.4, 0.6, 1, 1.5, 2 and 2.5. The effects of Lipozyme content were investigated with 5, 10, 25 or 50% dried Lipozyme IM20 (wt% of substrate). To test the effects of water activity, the water activity of dried Lipozyme was adjusted by rehydration in contact with the following saturated salt solutions for 6 days at 50°C (salt, water activity at 50°C): LiCl, 0.11; $MgCl_2$, 0.31; $Mg(NO_3)_2$, 0.45; NaCl, 0.74; and KNO_3, 0.85. Interesterification experiments were carried out by incubating the reaction mixture at the reaction temperature with shaking for 30 min prior to starting the reactions by adding the Lipozyme.

Analysis of the composition of technical palmitic and stearic acids and the liquid and solid fractions of crystallized ethyl stearate were carried out on the ethyl ester products by capillary gas chromatography (GC). A 30-meter SP2370 column with helium as the carrier gas was used at 200°C with injector and detector temperatures of 280°C.

Analysis of interesterification reactions was carried out in three ways. The free fatty acid content of the entire reaction mixture was determined by titration with 10 mM NaOH and phenolphthalein as indicator. Free fatty acids were converted to percent of the original triglyceride oil fraction of the substrate. The diglyceride content of the reaction mixture and the progress of the incorporation of stearate into the triglyceride oil were monitored by high-performance liquid chromatography with acetone/acetonitrile (70:30, vol. %) at 2 mL/min through a reversed-phase Lichrospher RP-18 column (250 mm × 4 mm, 5μ spheres; Merck, Darmstadt, Germany) and refractive index detection. Total mono-, di- and triglycerides were determined by capillary gas chromatography (CGC) on a 6-m DB-1 column with helium as the carrier gas and the following temperature program: Injector, 75°C, no heating; column, 80°C for 2 min, heated at 8°C/min to 170°C, then heated at 14°C/min to 350°C and held; detector, 350°C. Samples for the latter analysis were derivatized with MSHFBA [N-methyl-N-trimethylsilyl-heptafluor(o)-butyramide] purchased from Machery and Nagel (Düren, Germany). Results from the latter analysis were only used to confirm that small amounts of monoglycerides were formed and were not analyzed quantitatively.

The incorporation of stearate into sunflower seed oil was determined as previously presented (5–7). Diglycerides are presented below as wt% of triglycerides + diglycerides; free fatty acids are presented as percentages of the same. Because one triglyceride molecule can generate both a diglyceride molecule and a free fatty acid molecule, the sum of diglycerides + triglycerides is set to 100%, and free fatty acids are reported as percentages of the original triglyceride content of the substrate.

RESULTS

Ester synthesis reactions. The reflux temperature of the reaction mixture depended on the content of substrate or product at low ratios of solvent to fat (Fig. 1). In hexane, the reflux temperature was high when the ratio of solvent to product was low. The reflux temperature was lower in the presence of substrate and was depressed below the boiling point of hexane (69°C) at solvent/substrate ratios of 1–2 (vol/wt). The reflux temperature of petroleum ether containing substrate or product was lower than of hexane and depended less on the vol/wt ratio than in hexane at solvent/fat ratios above 1.5. The temperature of refluxing substrate mixtures was lower than the temperature of refluxing product mixtures. This may be due to the lower polarity of the product ethyl ester than the carboxylic acid, making the product more like the nonpolar solvent. Hexane/substrate ratios of about 2.5 or greater should be chosen to ensure that the reaction temperature remains close to the boiling point of the selected solvent. Petroleum ether/substrate ratios of about 1.5 or greater can be used to achieve the same effect. It is possible that the reflux temperature of petroleum ether may be less affected by the presence of fat because it boils over the entire range from 60 to 70°C.

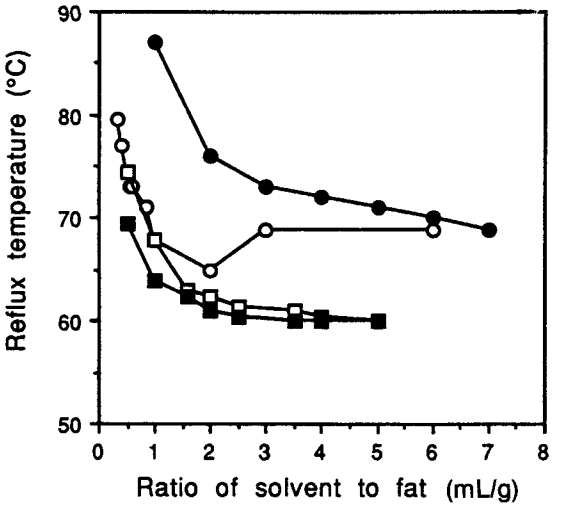


FIG. 1. Effects of solvent content on reflux temperature. Solvent was added to substrate (20 g stearic acid plus 5.1 mL dry absolute ethanol) or product (20 g ethyl stearate), and the reflux temperature was measured by immersing a thermometer in the refluxing solvent. Symbols: ○, stearic acid in hexane; ●, ethyl stearate in hexane; □, stearic acid in petroleum ether; ■, ethyl stearate in petroleum ether.

The reaction time needed to reach >99% conversion to ethyl esters decreased with increasing Lipozyme content up to 10% (Fig. 2). No decrease in reaction time was observed when the amount of catalyst was increased from 10 to 20%. Water removal was slower than the enzymatic reaction when 10% or more Lipozyme was used and, thus, water removal became rate-limiting. Because the reaction is reversible, it is better to operate under conditions where the rate of the enzymatic reaction is rate-limiting.

Scaling up was conducted by starting with 10 g stearic acid and increasing the amount of substrate stepwise by approximately doubling. The initial rate was low in the smallest scale (10 g) and high in the 80- and 150-g experiments, in which the 120-mL relux trap was used but was lower when 300 g of substrate was used (Fig. 3). The latter initial rate was about the same as the initial rates observed in all the experiments with the 450-mL reflux trap, which was constant regardless of substrate content or Lipozyme loading. Two different lipase loadings were used in these experiments. The initial rates of ethyl

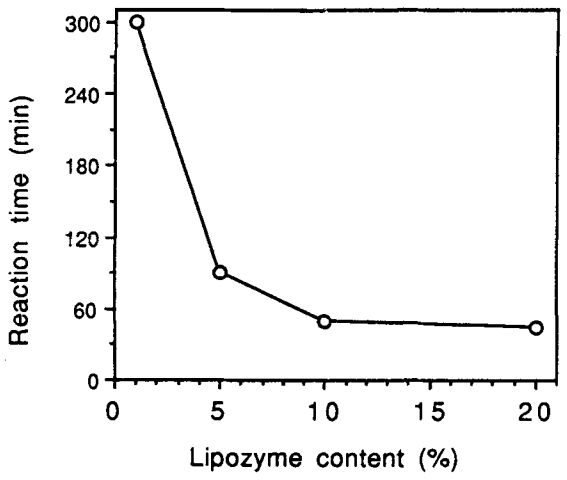


FIG. 2. Dependency of reaction time to reach >99% conversion on Lipozyme content in ester synthesis. Lipozyme IM60 was added to reaction mixtures consisting of stearic acid (40–50 g) and dried absolute ethanol (1.25 mol/mol stearic acid) in refluxing hexane.

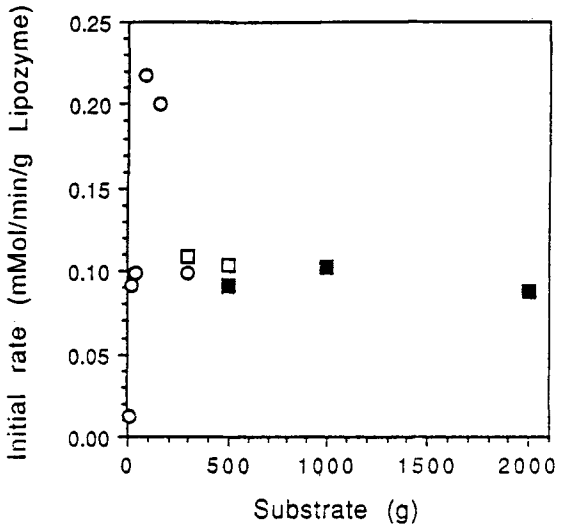


FIG. 3. Effects of the amount of substrate, the choice of Lipozyme and the volume of reflux trap on initial rates of ethyl stearate synthesis. Symbols: ○, Lipozyme IM60 with 120 mL reflux trap; □, Lipozyme IM60 with 450 mL reflux trap; ■, Lipozyme IM20 with 450 mL reflux trap.

stearate synthesis by these two catalyst preparations in the 500-g scale experiments were not greatly affected by the choice of Lipozyme. Water removal was probably rate-limiting, so there was no benefit to using the more highly active preparation in large-scale syntheses. As the scale of reaction was increased, the total time to bring the reaction to >99% completion became longer. When synthesis of ethyl palmitate was carried out, the progress of the reaction on a scale of 1 or 2 kg was almost identical in all respects to syntheses with stearic acid, including the initial rate (results not shown).

Two different reflux traps were used in the scaling-up procedure. The trap used for syntheses up to the 300-g scale has been described earlier (8). It has a trap volume of 120 mL. The reflux trap used for syntheses with 300 g or more of substrate had a trap volume of 450 mL. Large variations in initial rate were observed when the smaller reflux trap was used. It appears that the reaction volumes were optimal for that trap in the range of 75–150 g of acid substrate. The larger syntheses generated significantly larger volumes of water-rich phase, and water removal was again believed to be rate-limiting. For example, in the synthesis with the highest initial rate, the 80-g scale, the total volume of water-rich phase removed from the trap was 21.3 mL. In the synthesis with 1000-g, the total volume of water-rich phase removed was 289 mL. The latter reaction was probably more dependent on the water-removal process due to the large volume generated.

The progress of a typical large-scale synthesis of ethyl stearate (2-kg scale) is shown in Figure 4. The initial conversion was rapid. As the conversion reached about 80%, the reaction slowed. Conversion reached >99% in 4 h. The water content of water-rich phase increased as the level of conversion increased up to about 90%; then it started decreasing. At this point the flow of condensed reflux phase was directed over a column of molecular sieves (preconditioned as described in Materials and Methods) before being returned to the reaction vessel. As the content of water in the water-rich phase increased, the volume of the water-rich phase removed from the trap every 5 min decreased (results not shown). In a 1-kg synthesis, the synthesis was complete after 2.5 h. In a similar control experiment without the column of molecular sieves, the conversion reached >99%, but it took 3 h.

The operational stability of the Lipozyme preparation in 1-kg batch syntheses of ethyl stearate was excellent (Fig. 5). The initial rates were lowest in the first reaction studied and maintained a high level throughout the series. The half-life of Lipozyme is reported to be 1800 h under process conditions at 60°C (9). Our results have confirmed the operational stability of this enzyme preparation.

Interesterification reactions. Crystallization of the crude ethyl stearate almost completely eliminated the ethyl

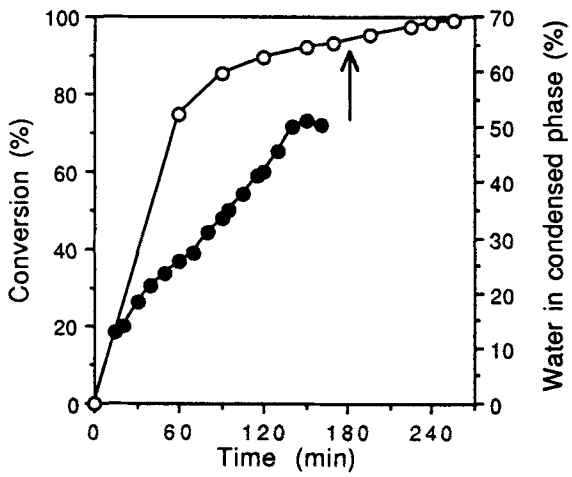


FIG. 4. Progress of ethyl stearate batch synthesis on 2-kg scale. Lipozyme IM 60 (100 g) was added to stearic acid (2 kg) dissolved in refluxing petroleum ether 60–70° (5 L). The arrow indicates when passage of the reflux solvent through a bed of molecular sieves (pretreated with ethanol) was started. Symbols: ○, conversion (%); ●, water content of water-rich phase collected in reflux trap.

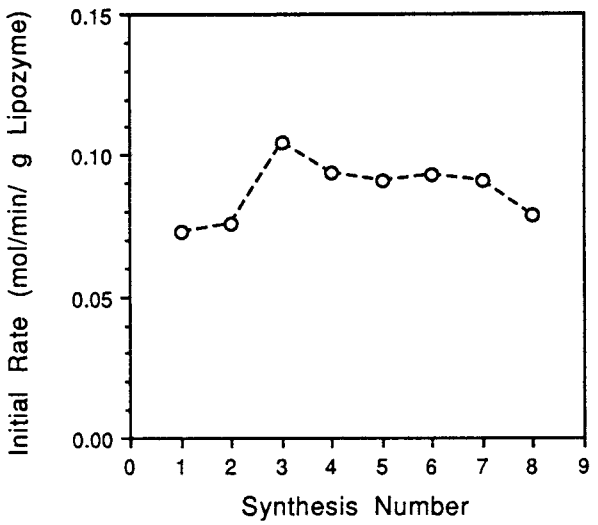
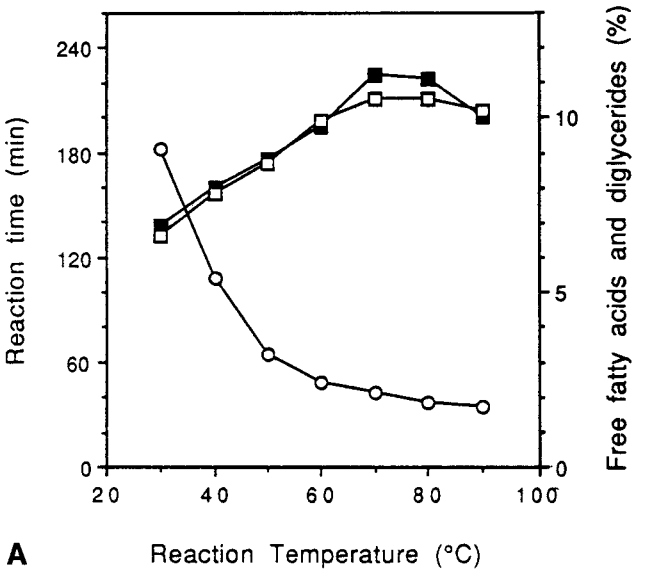


FIG. 5. Operational stability of Lipozyme IM20. Repeated 1-kg syntheses of ethyl stearate were catalyzed by a single batch of 25 g of Lipozyme IM20.



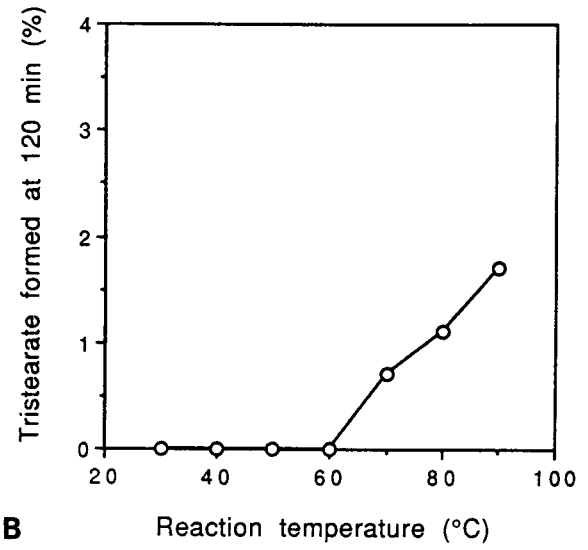


FIG. 6. A. Effects of reaction temperature on the time required to reach the interesterification reaction endpoint and the levels of diglycerides and free fatty acids present in the reaction mixture at the endpoint. B. Tristearate formation at 120 min at different temperatures. Symbols: ○, reaction time; □, free fatty acids at endpoint; ■, diglycerides at endpoint.

palmitate from the ethyl stearate product (Table 1). The liquid fractions that were decanted from the crystallized ethyl stearate were enriched in ethyl palmitate, ethyl arachidate and ethyl behenate (results not shown).

The reaction time decreased with increasing temperature in the range 30–90°C (Fig. 6A). The free fatty acid and diglyceride contents of the reactions were almost identical, which was due to the low levels of monoglycerides produced in the reaction (results not shown). These by-product levels at the endpoint increased with increasing temperature in the range 30–70°C and did not change above 70°C. No tristearate was observed at the endpoint at any reaction temperature. However, tristearate was observed at 120 min in reactions conducted above 60°C (Fig. 6B). The tristearate level at 120 min increased with temperature, which is the expected pattern if tristearate formation occurs by acyl migration of reaction intermediates and because acyl migration is accelerated by heat (6). No tristearate was observed over the entire duration sampled for reactions at 30–60°C (240 min in reactions conducted at 40–60°C; 420 min at 30°C). Based on these findings, a reaction temperature of 50°C was chosen for investigation of the effects of other experimental variables.

Comparison of the reaction times at 70–90°C with small-scale acidolysis reactions between palmitic acid and triolein shows that the interesterification reaction is 5–6 times faster than the corresponding acidolysis reaction (7). In addition, the reaction time of the slowest interesterification reaction (at 30°C), which was complete in 182 min, was as short as the fastest acidolysis reaction (at 90°C) in the previous work. In acidolysis, trisaturated triglyceride formation also increased with the reaction temperature in the range 70–90°C and was somewhat higher than in the present work.

The present results show that the interesterification reaction can be conducted over a wide temperature range when ethyl esters are used as the acyl donor. This gives the operator a choice between large reductions in reaction time as compared to the use of acid substrates by operating at high temperatures, or operating at lower temperatures to suppress diglycerides and free fatty acid levels in the product without unreasonably long reaction times. The latter alternative is believed to result in greater operational stability of the lipase preparation and reduced acyl migration.

Substrate ratio. The effects of substrate ratio on the reaction time required to reach the endpoint and on the amount of free fatty acids and diglycerides formed are depicted in Figure 7. Reaction times decreased with increasing ratio of acyl donor to triglyceride oil. At substrate ratios of ester/triglyceride of 0.4 and 0.66, the reaction did not reach the endpoint in 240 min. The free fatty acid content increased slightly with increasing ethyl stearate content. More diglycerides were generated at higher ester/oil ratios. No trisaturated triglycerides were detected at any

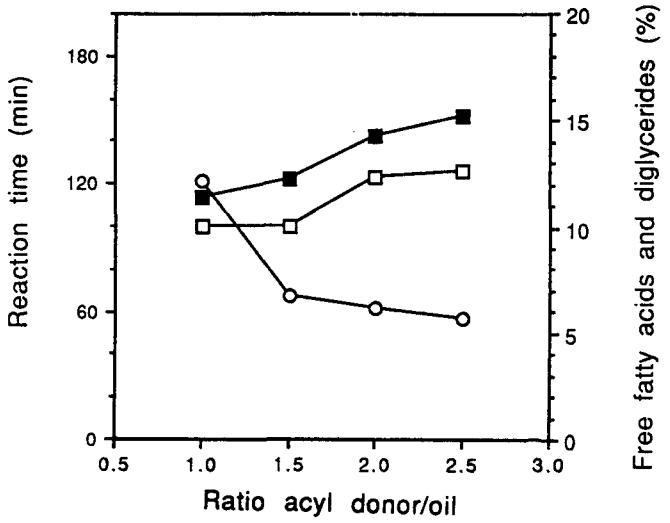


FIG. 7. Effects of weight ratio of ethyl stearate to triglycerides on the time required to reach the interesterification reaction endpoint and the levels of diglycerides and free fatty acids present in the reaction mixture at the endpoint. The weight ratios correspond to the following molar ratios, assuming the molecular weight of sunflower seed oil is equal to that of triolein (wt ratio/molar ratio): 1:2.7; 1.5:4.0; 2:5.4; 2.5:6.7. Symbols: O, reaction time; □, free fatty acids at endpoint; ■, diglycerides at endpoint.

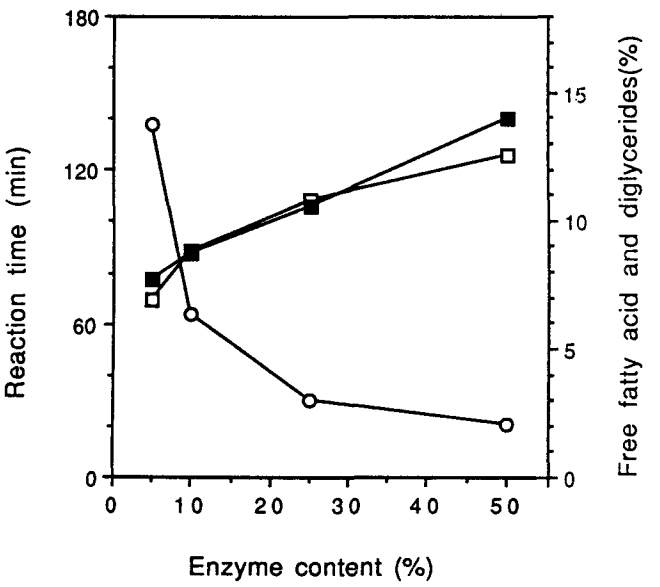


FIG. 8. Effects of Lipozyme content on the time required to reach the interesterification reaction endpoint and the levels of diglycerides and free fatty acids present in the reaction mixture at the endpoint. Symbols: O, reaction time; □, free fatty acids at endpoint; ■, diglycerides at endpoint.

substrate ratio for the duration of sampling (240 min). In small-scale acidolysis reactions, longer reaction times were required for mixtures with low ratios of acid to triglyceride, but the reaction time was not dependent on the substrate ratio at the substrate ratios reported in the present work (7).

Lipozyme content. The Lipozyme content was varied from 5–50% at 50°C. The reaction time decreased and free fatty acid and diglyceride content increased with increasing Lipozyme content (Fig. 8). Reactions where 50% Lipozyme was used were not 10 times faster than reactions with 5% Lipozyme. The free fatty acid and diglyceride content of the reaction mixture at the endpoint increased with increasing Lipozyme content and, therefore, with decreasing reaction time. No tristearate formation at the endpoint occurred in any of the reactions. The lowest free fatty acid content was observed with 5% Lipozyme and was the same as that obtained with 10% Lipozyme at 30°C. The presence of more catalyst accelerates the reaction and increases the content of free fatty acids and diglycerides in the reaction mixture. This has been observed previously (10). Small-scale control experiments were carried out with 600 mg commercial ethyl stearate and 300 mg pure triolein (results not shown). Almost identical reaction times were observed on the 900-mg and 100-g scales.

Water activity. The water activity of Lipozyme was adjusted by drying followed by equilibration with saturated salt solutions. Because the half-time of equilibration of Lipozyme is about 0.5 d, a 6-d equilibration period was considered adequate (11). The substrate was not equilibrated because the water content of substrate oil is insignificant as compared to the contribution of Lipozyme of controlled water activity to the total water content of the reaction mixture (7). Intermediate water activities produced the shortest reaction times (Fig. 9). As expected,

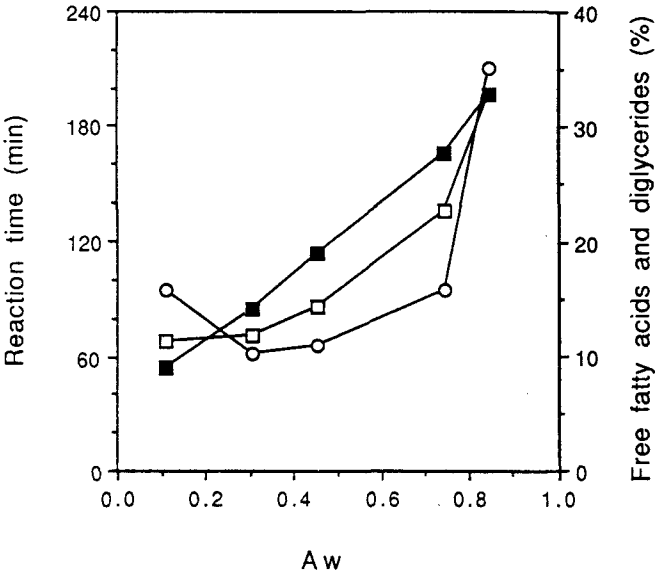


FIG. 9. Effects of water activity of Lipozyme on the time required to reach the interesterification reaction endpoint and the levels of diglycerides and free fatty acids present in the reaction mixture at the endpoint. Symbols: O, reaction time; □, free fatty acids at endpoint; ■, diglycerides at endpoint.

the free fatty acid and diglyceride contents increased with increasing water activity of the Lipozyme. No tristearate was formed at the reaction endpoint at any water activity. The reaction conducted with dried Lipozyme was more rapid and resulted in fewer free fatty acids and diglycerides than all the reactions with equilibrated Lipozyme. Activity losses may have occurred after 6 d of equilibration at 50°C. Diglyceride levels were lower than those obtained

in small-scale interesterification reactions between triolein and ethyl palmitate (7). However, those reactions were conducted in the presence of saturated salt solutions of the same water activity as used for pre-incubation of Lipozyme, which provided more water for the reaction and higher diglyceride levels. When the initial rate of ester synthesis was correlated with the water activity of the Lipozyme catalyst, the highest initial rates were obtained at intermediate water activities (11).

Reflux trap interesterifications. The interesterification reaction was conducted with 100 g of substrate and 10 g dried Lipozyme IM20 in 300 mL rapidly stirred refluxing pentane (b.p., 36°C). The free fatty acid and diglyceride levels present in the product at the endpoint were reduced when this method was used. Despite the increased reaction volume, the reaction time was close to what would be predicted by interpolation from Figure 6 (Fig. 10). The free fatty acid content of the reaction mixture at the endpoint was low, 5.1%. The diglyceride content decreased to 4.4% during the course of the reaction, which is an expected result of conducting the reaction under drying. When dry activated molecular sieves were added to the reflux trap, the reaction time was similar, but the free fatty acid content at the endpoint was 6%. The diglyceride level at 20 min was the same for both reactions, but when sieves were added the diglyceride content was reduced to 3.3% at the endpoint. This is what would be expected if the molecular sieves absorbed ethanol released from ethyl stearate:

$$\text{ethyl stearate} \rightleftharpoons \text{stearic acid} + \text{ethanol}$$

$$\text{diglyceride} + \text{free fatty acid} \rightleftharpoons \text{triglyceride} + \text{water}$$

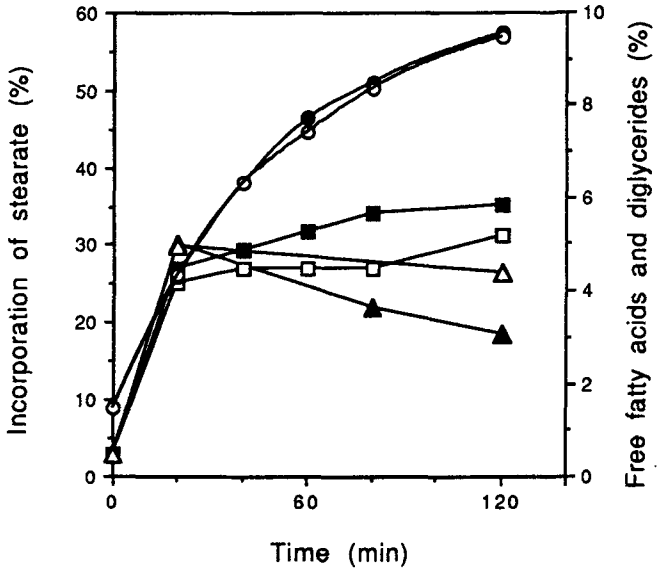


FIG. 10. Progress of interesterification reaction conducted with continuous drying with a reflux trap. Substrate (100 g) was dissolved in 300 g *n*-pentane and the reaction was started by adding 10 g vacuum-dried Lipozyme IM20 (Aw = 0.07). Symbols: ○●, incorporation of stearate into triglycerides of sunflower seed oil; □■, free fatty acids in reaction mixture; △▲, diglycerides in reaction mixture. Open symbols, refluxing pentane without drying; and filled symbols, refluxing pentane with continuous drying.

Because activated molecular sieves remove both water and ethanol (8), both reactions are driven to the right, yielding lower diglyceride concentrations and higher free fatty acid concentrations than in the reaction conducted without molecular sieves. Water removal has been used successfully to reduce the level of diglycerides in the product to a level below that of the starting material in interesterification (12).

DISCUSSION

The interesterification of fats is always accompanied by diglyceride formation, as diglycerides are unavoidable intermediates in the reaction. Diglyceride levels of 8.8 (13), 10 (14–16) and 16% (17) have been reported. Lower diglyceride levels have been obtained with cells than when immobilized lipase is used (13,14). The increase in production of diglycerides with increasing water content in lipase-catalyzed interesterifications is widely reported (10, 12,14,16,18–21). More diglycerides are also formed with ester substrates than with free fatty acids (7,18,22,23). One explanation for this phenomenon is thermodynamic; acidolysis may be less prone to hydrolysis because the fatty acid substrate shifts the reaction equilibrium toward triglyceride synthesis, which can also reduce the amount of diglycerides in the reaction mixture (18,24). It also has been observed that diglyceride formation in packed-bed reactors is less than in batch reactors (17). This is due to dehydration of the lipase, which occurs in the front of a bed reactor until the water content of the reaction mixture has been reduced to about half of saturation (0.03%) (10).

Most by-products are formed in the first part of the reaction (7,14,19). It appears that in this first part of the reaction, the lipase reduces the water content of the reaction mixture to a level optimum for interesterification reactions. Therefore, re-using the lipase without washing or rehydrating results in dramatically reduced hydrolysis products (13,19). In continuous processes, the use of dry oils has been observed to reduce catalytic activity by dehydrating the lipase preparation, so oils are often hydrated before passage through a column (14,19).

The content of diglycerides in a reaction mixture also can increase by acyl migration of reaction intermediates to form 1,3-diglycerides, which are unable to deacylate the enzyme (6,14). Thus, 1,3-diglyceride levels in Lipozyme-catalyzed interesterifications increased slowly with reaction time, while 1,2-diglyceride levels actually decreased over a 30-h reaction period (25). The primary mechanism of formation of 1,3-diglycerides has been shown to proceed by lipase-catalyzed hydrolysis of 1,2(2,3)-diglycerides to form unstable 2-monoglycerides (26). The 2-monoglyceride isomerizes to form a 1(3)-monoglyceride, which is re-esterified by the lipase. Thus, excess water, which causes this hydrolysis, increases the diglyceride content of the reaction mixture by generating a by-product unable to interact with a 1,3-positionally specific lipase.

The use of ethyl ester substrates conveys certain advantages, such as the ability of operating at lower temperatures to preserve enzymatic activity and reduce heat-catalyzed isomerization of reaction intermediates. Because acid can also catalyze this isomerization, replacing acids with esters as acyl donors also removes a potential catalyst for acyl migration. Purification of ethyl stearate

by crystallization was also simple in this work. Some of the water on a lipase preparation can be replaced with ethanol, but the proper level of addition is critical (27).

The dehydration of Lipozyme with repeated use in 500-g acidolysis batch reactions has been observed; the amount of free fatty acid formed decreased from 6.4 to 1.2% as the water content of Lipozyme decreased from 6.2 to 0.9% with repeated use (23). This led to decreased incorporation of polyunsaturated fatty acid into product triglycerides. Activity was fully recovered with respect to incorporation of eicosapentaenoic acid and almost fully recovered with respect to docosahexaenoic acid when Lipozyme was rehydrated (23). Similar dehydration has been observed in columns, which is attributed as the cause for reduced free fatty acid formation in column reactors (10,17). In a recent application, a water activity sensor was used to monitor the water activity of substrates of lipase-catalyzed interesterification. The product composition also was monitored, and a feedback control device was used to maintain the optimal water content for suppression of excessive diglyceride formation and a constant composition of product triglycerides (28).

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