# Triglyceride Interesterification by Lipases. 1. Cocoa Butter Equivalents From a Fraction of Palm Oil

**Scott Bloomer, Patrick Adlercreutz*** and **Bo Mattiasson**
Department of Biotechnology, Chemical Center, University of Lund, P.O. Box 124, S-221 00, Lund, Sweden

**Twelve commercially available triacylglycerol lipase preparations were screened for their suitability as catalysts in the interesterification of palm oil mid fraction and ethyl stearate to form a cocoa butter equivalent. Five fungal lipase preparations were found to be suitable. The hydrolytic activity of the commercial lipase preparations was tested with sunflower seed oil and was independent of their interesterification activity. The operational stability of three of the preparations most suited for production of cocoa butter equivalents was examined. The amount of a commercial lipase preparation loaded onto a support was surveyed for optimum short-term catalytic activity.**

**The influence of solvent concentration on the reaction rate and the purity of the product was examined at two temperatures. The optimum solvent concentration at 40°C was 1-1.5 grams of solvent/gram of substrate; at 60°C, the rate of interesterification diminished and the purity of the product decreased with increasing amounts of solvent.**

**Four of the commercial lipase preparations found to be suitable interesterification catalysts were immobilized on five supports and their ability to catalyze the interesterification of a triglyceride and palmitic acid or ethyl palmitate was measured. The choice of support and substrate form (esterified or free fatty acid) greatly affected the catalytic activity. Some preparations were more affected by the choice of support, others by the form of the substrate. No preparation yielded maximum activity on all supports, and no support was found which produced an immobilized enzyme preparation of high activity with every commercial lipase preparation. Caution is advised in transferring observations about the suitability of a support from tests on one commercial enzyme preparation to others; individual testing is required.**

KEY WORDS: Cocoa butter equivalent, interesterification, lipase screening, operational stability, solvent concentration, support screening.

Triacylglycerol lipases (EC 3.1.1.3) can be used in several ways in the modification of triglycerides. In an aqueous medium with an emulsified triglyceride substrate, hydrolysis is the dominant reaction, but in organic media esterification and interesterification reactions can become predominant; this is now a well established application in the field of bioorganic chemistry (1-4). Lipases from different sources display hydrolytic positional specificity and some fatty acid specificity (5). The positional specificity is retained when lipases are used in organic media (6), but little information is available about their fatty acid selectivity in organic media.

One application of the lipase catalyzed interesterification of triglycerides which has been studied is the preparation of cocoa butter equivalents. Normally the starting material is palm oil mid fraction (POMF) and the purpose of the lipase catalyzed reaction is to introduce more stearate into the triglycerides of the POMF. The main component of palm oil mid fraction is the triglyceride 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), which can be converted to 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POSt) and 1,3-distearoyl-2-oleoyl-glycerol (StOSt) by the interesterification reaction. The result is a fat with a triglyceride composition resembling cocoa butter (CB) which can be used as a cocoa butter equivalent (CBE) in the chocolate and confectionary industry. As it is the fatty acids in position 1 and 3 which should be replaced, the 1,3-specific lipases are the enzymes of choice. The reaction medium normally consists of a mixture of the triglyceride to be converted and the stearate component, either as free acid or an ester, in a nonpolar organic solvent. In practical applications the possibility of reusing the enzyme is important for reasons of process economy; immobilization of the lipase is necessary to facilitate enzyme recovery for re-use and to set up continuous processes. Immobilization for use in non-aqueous systems can be done simply by mixing an aqueous solution of the enzyme with a suitable support material and removing the water at reduced pressure, after which small amounts of water are added to activate the enzyme. Lipase preparations are available from several sources and manufacturers. The activity, stability, and purity of these preparations is subject to large variations. Despite the high levels of non-protein material present in commercial lipase preparations, they can express good activity without further purification, and therefore the characterization of their use without purification is needed if they are to be useful outside the pure enzymologist's laboratory. To our knowledge, the enzymatic interesterification reaction for the production of cocoa butter equivalents is not yet carried out on a full industrial scale, despite much research effort.

There are many factors which influence the performance of an enzyme-based immobilized preparation. Some of the most important of these factors are the solvent, the support material and the water content (7,8). In the present investigation the emphasis is on screening in order to be able to use commercial enzyme preparations as received, without further purification, in catalyzing interesterification reactions for the formation of cocoa butter equivalents. The protein content, interesterification activity, and hydrolytic activity of the commercial lipase preparations (CLP's) was determined and the operational stability of three of the best interesterification catalysts was examined. The influence of the amount of enzyme immobilized on a support and the amount of solvent present was investigated. The four enzymes with the highest interesterification activity were immobilized on five different supports and tested for their ability to catalyze an interesterification reaction with a different substrate.

*To whom correspondence should be addressed.

## EXPERIMENTAL PROCEDURES

*Materials.* Interesterification assays were conducted on substrates consisting either of ethyl stearate (Sigma Chemical Co., St. Louis, MO) with a bleached, fractionated, and deodorized palm oil mid fraction (donated by Karlshamns AB, Karlshamns, Sweden) or palmitic acid, methyl palmitate (both from BDH, Poole, England), or ethyl palmitate (Sigma) with triolein (Serva, Heidelberg, W. Germany). A cocoa butter standard was donated by Karlshamns AB. Assays of lipolytic activity were made with sunflower seed oil (Sigma) as substrate. Comparisons of supports were conducted on celite (BDH), Hyflo Supercel (donated by Karlshamns AB), low-density polyethylene powder and polyamide powder PA6 ("Accurel" materials, both donated by Akzo, Obernburg, W. Germany), and macroporous anion exchange resin. This resin was obtained by boiling a preparation of Lipozyme (donated by Novo Industri A/S, Denmark) in three changes of water, followed by autoclaving and drying at 110°C. Interesterification tests confirmed the absence of activity of the resin after this treatment. Propanenitrile for HPLC was obtained from Fluka (Buchs, Switzerland).

Commercial enzyme preparations (triacylglycerol lipases, EC 3.1.1.3.) used in this study were: Lipozyme (donated by Novo Industri A/S), Enzymatix F3, F4, and F8, Amano D, F, and N (all donated by Karlshamns AB), lipase from *R. arrhizus* (donated by Gist-brocades, Holland), Röhm lipases 2212E and 7023C (donated by Röhm GmbH, Darmstadt, West Germany), and lipases from *C. cylindracea* and Type 2 crude pork pancreas (Sigma). All commercial lipase preparations were used as received, without further purification. All lipases tested except that from *C. cylindracea* are specific for hydrolysis and esterification of fatty acids in the 1 and 3 positions of glycerides. Bovine serum albumin was from Sigma.

*Procedures.* Commercial lipase preparations (CLP's) were immobilized on supports by dissolving the enzymes in 50 mM phosphate buffer solution at the pH optimum reported by the manufacturer and adding the enzyme solution to the support while stirring. In most cases this was pH 7, 10 μL/mg CLP, but Amano D was immobilized at pH 6 and Röhm 2212E was immobilized with 2 μL buffer/mg enzyme. Quantities of support and buffer were chosen so that the entire liquid phase was absorbed onto the support and no separate phase was formed; normally 1 mL of solution was used per gram of support. The resulting lipase catalyst preparations (referred to as "immobilized preparations" below) were dried under vacuum to constant weight.

The lipolytic activity of enzymes was tested with sunflower seed oil. The substrate was prepared by emulsifying 500 mg sunflower seed oil on a vortex mixer with 9.5 mL sodium chloride solution (0.89% NaCl w/v) and 500 mg gum arabic (added slowly to the liquids during vortex stirring) (9). Vortex stirring was continued at room temperature for one minute and the emulsion was used immediately. The assay mixture for lipolytic activity was made up of 5 mL of substrate emulsion, 50 μL sodium deoxycholate (10 mM) and enough 0.89% NaCl solution to bring the total volume to 10 mL. The pH was adjusted to 7 by the addition of 10 mM NaOH, and 50–200 μL of lipase solution (10–25 mg lipase dissolved in 1 mL 0.89% sodium chloride solution) was added. The continuously stirred mixture was incubated at 40°C in a jacketed incubation vial. The release of free fatty acids was monitored by continuous titration with 10 mM NaOH with a 665 Dosimat automatic burette (Metrohm, Switzerland).

The interesterification activity of CLP's was determined in two ways. The stearate system consisted of a mixture of palm oil mid fraction and ethyl stearate (1 gram, 1:2 by wt.) dissolved in 3.5 mL n-heptane and incubated at 40°C for 30 min. Vacuum-dried immobilized preparation (100 mg [=10 mg CLP on 90 mg celite]) was activated by the addition of water (10 μL, 10% wt/wt) and added to the substrate mixture; the reactions were carried out in 10-mL stoppered glass vials with reciprocal shaking at 150 RPM. This system was used to measure interesterification activities of CLP's and the effects of changes in enzyme loading. Operational stability of the CLP's was tested using a reduced quantity of biocatalyst in 4 g of substrate (2 g palm oil mid fraction and 2 g ethyl stearate in 6.8 mL water-saturated n-hexane), and the reaction mixture was withdrawn and replaced with fresh substrate every 24 hr. The palmitate system used for the comparison of supports consisted of a 1 gram mixture of Serva triolein and either palmitic acid (10), methyl palmitate, or ethyl palmitate in a molar ratio of 1:3. CLP (10 mg) was immobilized on 90 mg support and activated by the addition of 5% (wt% of immobilized preparation) water. Water-saturated n-heptane (3.5 mL) was added and the reaction was carried out at 40°C in stoppered 10 mL glass vials with reciprocal shaking at 150 RPM.

Samples were analyzed on a Shimadzu HPLC (Shimadzu Scientific Instruments, Columbia, MD) using a LiChrosorb RP-18 (5 μm) column (250 × 4 mm) eluted at 30°C with propanenitrile (1 mL/min) and monitored with an RI detector (11). Chromatograms were processed on a Shimadzu CR4A integrator.

The protein content of the commercial lipase preparations was determined with the Biuret method with bovine serum albumin as the standard (12).

## RESULTS AND DISCUSSION

*Lipase screening.* The protein content of 11 commercial lipase preparations (CLP's) available in powder form was determined (Table 1). Large variations in the protein content were noted. The CLP's were immobilized on celite, and one available as an immobilized preparation (Lipozyme) was used after drying and rehydrating with a known amount of water. Information about the protein content of this preparation is unavailable, so values for the hydrolytic and interesterification activity for this preparation are based on the entire weight of Lipozyme used (Table 1). Automatic titration of free fatty acids released by hydrolysis of sunflower seed oil was used to determine the hydrolytic activity of the CLP's. In this assay the highest specific activity was observed with the *Rhizopus arrhizus* lipase preparation from Gist-brocades, which was more than ten times as active as the CLP with the lowest activity (Amano N). High protein content in the CLP's does not insure high activity; the four CLP's which contained the most protein exhibited the lowest hydrolytic activity.

Although the hydrolytic activity values give some information on the usefulness of the CLP's, their interesterification activity cannot be predicted from their

TABLE 1

**Lipase Screening**

| Name | Source | Protein % | Hydrolysis Units/g protein | Transesterification % conv to CB[a]/ mg CLP × hr | Transesterification % conv to CB/ mg protein × hr | Trisaturated glycerides[b] % |
|---|---|---|---|---|---|---|
| Lipozyme (Novo) | *Mucor miehei* | .nd | 21.8[c] | .427[c] | — | 12.4 |
| Gist-brocades | *Rhizopus arrhizus* | 27.0 | 1319 | .625 | 2.315 | 11.3 |
| Sigma | Pig pancreas | 24.7 | 1150 | — | — | — |
| Enzymatix F3 | *Rhizopus* sp. | 37.2 | 782 | .435 | 1.169 | 13.5 |
| Amano F | *Rhizopus javanicus* | 49.0 | 594 | — | — | — |
| Enzymatix F8 | *Aspergillus niger* | 23.9 | 548 | — | — | — |
| Amano D | *Rhizopus delemar* | 20.8 | 423 | — | — | — |
| Röhm 2212E | Fungal origin | 32.6 | 353 | .625 | 1.917 | 10.1 |
| Enzymatix F4 | *Rhizopus* sp. | 68.0 | 285 | .555 | 0.816 | 11.9 |
| Sigma | *Candida cylindracea* | 63.8 | 155 | — | — | — |
| Röhm 7023C | Pig pancreas | 71.5 | 150 | — | — | — |
| Amano N | *Rhizopus niveus* | 78.0 | 122 | — | — | — |

Interesterification was performed with commercial lipase preparations immobilized on celite (30–80 mesh). Water (5 μL) was added to the immobilized preparation (100 mg) prior to its addition to the reactor. Lipozyme was used after vacuum drying; high activity was expressed without additional water. The substrate used was 1 gram of a 1:2 (wt:wt) mixture of palm oil mid-fraction:ethyl stearate in 3.5 mL of water-saturated n-heptane at 40°C. Hydrolysis of sunflower seed oil was carried out at pH 7 and 40°C on a pH-stat.

[a]The figures denote the amount of incorporation of stearate into palm oil mid fraction to form monounsaturated triglycerides per mg commercial lipase preparation and hour. The stearate content of cocoa butter (CB) was defined as 100%.

[b]Percent of triglycerides in the reaction mixture which contained only saturated fatty acids when the incorporation of stearate into the monounsaturated triglycerides in the reaction mixture was equivalent to that found in cocoa butter. Stearoyl-dioleoyl glycerol (StOO) is included, as the HPLC method was unable to resolve this compound from tripalmitate.

[c]Activity expressed in units per gram of Lipozyme, which includes an unspecified amount of protein bound to a support.

hydrolytic activity (13,14). The interesterification of ethyl stearate and a bleached, deodorized and further refined palm oil mid fraction (POMF) was used to determine this activity. The measure of interesterification activity used was the incorporation of stearate into the monounsaturated triglycerides of the product, denoted Σ and defined by the formula Σ = (% POS + 2 [% SOS])/2(% POP + % POS + % SOS) × 100. Percentage values were calculated from the total triglyceride content of the samples.

The refined POMF substrate we used has a Σ value of 10.4; the desired product, cocoa butter, has a Σ value of 57. Reactions were allowed to continue until the level of stearate incorporated into the monounsaturated triglycerides (POP + POSt + StOSt) of the palm oil fraction was equal to that found in cocoa butter (Σ = 57). Interesterification rates were calculated from the amount of time per milligram of CLP and protein required to reach this level. The rates are a measure of the average speed of incorporation of stearate during the reaction and reflect the catalytic efficiency and the specificity of the enzyme for stearate. All of the CLP's immobilized on celite were able to catalyze the interesterification to a degree, but only five were able to incorporate enough stearate to repeatedly reach the Σ value of cocoa butter. These are: Lipozyme, Enzymatix F3, Enzymatix F4, Gist-brocades *R. arrhizus* lipase, and Röhm 2212E (Table 1). When first received, the Amano lipases displayed good or very good interesterification activity; subsequent immobilized preparations and repeated tests of the first biocatalyst preparations showed much lower activity, despite careful storage at 4°C over dessicant. There was no clear correlation between the activities observed for the lipases in the hydrolysis and interesterification reactions, which is in agreement with earlier observations (13,14). The interesterification activity of the CLP's is independent of their protein content. Variations in relative hydrolytic/interesterification activities between immobilized preparations may be due to differences in ease of inactivation of the enzymes in the immobilization process, and to differences in the suitability of the support used (see below). Sunflower seed oil has a higher percentage of polyunsaturated (linoleic) fatty acids than palm oil mid fraction, so the fatty acid specificity of the lipases may play a role in the observed activities.

Several of the immobilized preparations which were unable to incorporate enough stearate to reach Σ = 57 catalyzed interesterification of the medium chain length fatty acids of POMF in the presence of ethyl stearate, and produced fat mixtures with triglyceride contents similar to tests in which enzymes were allowed to react with palm oil mid fraction without stearate (data not shown). It is well known that the fatty acid specificity of lipases varies from species to species (15), and this is probably the reason for these differences. This kind of result would occur if the specificity of the enzyme allowed it to catalyze reactions with the triglycerides of shorter chain length fatty acid in POMF but precluded interaction with ethyl stearate.

The formation of trisaturated glycerides occurs in the reaction mixture and is believed to be due to the presence of saturated fatty acids in the 2-position of the palm oil mid fraction. Formation of higher quantities of trisaturated triglycerides may also be due to acyl migration of diglycerides formed from the triglycerides in the reaction mixture. This process allows the unsaturated fatty acids originally occupying the 2-position of diglycerides to be hydrolyzed by a 1,3 specific lipase; the saturated

monoglycerides formed can be acylated with saturated fatty acids, forming disaturated diglycerides which may then be acylated to form trisaturated triglycerides (1). The formation of trisaturated glycerides is an undesirable side reaction in this system because it reduces the suitability of the product by increasing the amount of high melting material of the cocoa butter equivalent so that fractionation is necessary (16). Our analysis by HPLC of a cocoa butter standard indicated the presence of about 7% trisaturated glycerides. Macrae reported that the trisaturated glyceride content of cocoa butter is 2% (2); the HPLC method used in this study was not able to separate one of the trisaturated glycerides, tripalmitate (PPP), from the diunsaturated triglyceride stearoyl-dioleoyl-glycerol (StOO), and it is possible that part of the PPP content measured was StOO. The amount of trisaturated glycerides observed in these experiments was higher than that found in cocoa butter and varied little when different CLP's were used. The trisaturated glycerides formed were primarily palmitoyl-distearoyl-glycerol (PStSt), dipalmitoyl-stearoyl glycerol (PPSt), and tristearate (StStSt), although some increase in the amount of PPP + StOO was usually observed. Furthermore, the formation of trisaturated glycerides was not dependent on the interesterification rate, the hydrolysis rate, or the protein content of the CLP's (Table 1).

*Operational stability.* The operational stability of three of the most active CLP's was studied. Small amounts of CLP's were used in this experiment in order to detect any inactivation. The activity of the Gist-brocades preparation of lipase from *R. arrhizus* started at a higher level (expressed as the Σ level reached in 24 hr), but decreased steadily (Fig. 1). Both Lipozyme and the Röhm 2212E lipase preparation maintained a high level of activity throughout the period illustrated, and therefore these two preparations can be expected to yield better productivity in long-term use.

*Enzyme loading.* The effectiveness of immobilized preparations of different loads of a commercial enzyme

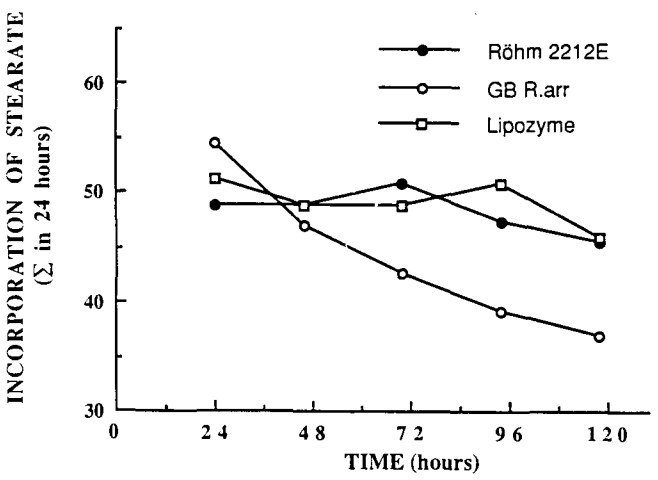


**FIG. 1. Operational stability of three commercial lipase preparations (CLP's). Substrate (2 g palm oil mid fraction and 2 g ethyl stearate in 6.8 mL water saturated n-hexane) was replaced every 24 hr. Weights of CLP's used (in wt % of substrate): Lipozyme, 1%, no water added; Röhm 2212E and *R. arrhizus* preparations, 5%, with 5% (wt % of immobilized preparation) water added. The reactions were carried out at 40°C with shaking at 150 RPM.**

**TABLE 2**

**Dependency of the Rate of Interesterification on the Amount of Röhm 2212E Lipase Preparation on Celite**

| Wt CLP in 100 mg immobilized preparation (mg) | Incorporation of stearate (Σ) |
|---|---|
| 5 | 21 |
| 10 | 51 |
| 20 | 51 |

Various amounts of commercial lipase preparation were immobilized by adsorption after dissolving in 20 mM phosphate buffer, pH 7, 5 mL/g CLP, and drying *in vacuo* onto celite to form an immobilized preparation. The immobilized preparation (100 mg) was activated by the addition of 5% (wt. %) water. Substrate: palm oil mid fraction (333 mg) and ethyl stearate (667 mg) in 3.8 mL water-saturated n-heptane at 40°C with reciprocal shaking at 150 RPM. Incorporation of stearate was measured at 6 hr.

preparation on celite was studied. The highly active Röhm 2212E lipase preparation was immobilized in various quantities on celite and the incorporation of stearate into palm oil mid fraction was measured. No increase in catalytic rate was observed with an enzyme load greater than 10% (by wt.) of immobilized preparation (Table 2). At higher loads the extra enzyme was not used effectively, probably because part of it became inaccessible to the substrate. It has earlier been observed that the location and access of an immobilized enzyme to the organic phase exerts a large effect on the observed activity (17-19). However, it is possible that the operational stability of highly loaded preparations will be higher, which means that the immobilized preparation may be used for a longer time without losing activity.

*Solvent concentration.* The influence of solvent concentration on the rate of interesterification and the formation of saturated triglycerides was studied at 40 and 60°C with Lipozyme, a thermostable enzyme. At 40°C, the rate of interesterification is highest at levels of solvent between 1 g and 1.5 g/gram of substrate (Fig. 2a). The substrate alone is not fully molten at 40°C, and the addition of solvent decreased the viscosity of the reaction mixtures visibly. The lower rate observed with less solvent was due to the difficulty of catalyzing interesterifications on solid fats (19). The conversion rate obtained with Lipozyme was higher at 60°C than at 40°C. At 60°C the substrate mixture is fully liquid; the reaction rate was high and the formation of saturated triglycerides was minimal without solvent. The addition of solvent (heptane) in excess of 0.5 g/g substrate caused a decrease in reaction rate, while the addition of solvent at levels of 0.5 g/g substrate and above caused an increase in the amount of trisaturated glycerides formed. Substrate dilution can be expected to slow the rate of catalysis as the solvent concentration increases. In addition, as the amount of solvent was increased, the amount of trisaturated glycerides formed increased (Fig. 2b). Formation of trisaturated glycerides by acyl migration is clearly favored by the lower rate of enzymatic catalysis. Consequently, it is beneficial to operate the process at 60°C without solvent, as under these conditions a high conversion rate was observed and the formation of trisaturated triglycerides was minimal.

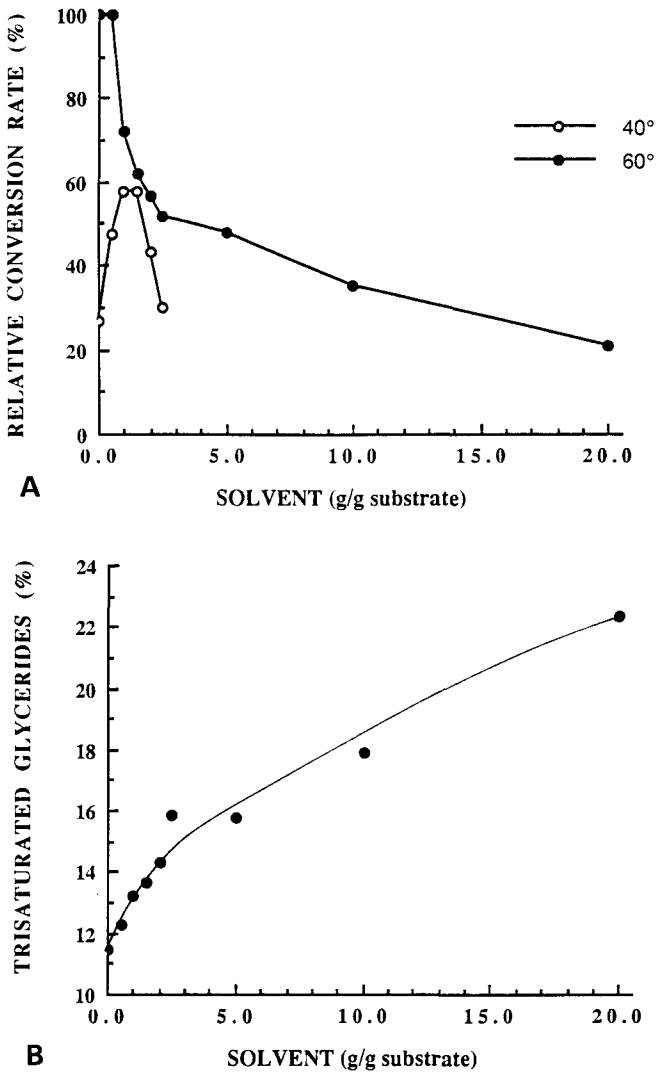


**FIG. 2. Dependency of the rate of conversion to cocoa butter equivalent at 40°C and 60°C (a) on the amount of n-heptane present, and the formation of trisaturated glycerides at 60°C (b). Substrate: palm oil mid fraction:ethyl stearate, 1:2 by wt. Vacuum dried Lipozyme was used without additional water; solvent was saturated with water. The relative conversion rate at 100% indicates the rate of incorporation of stearate into palm oil mid fraction to reach the level of stearate found in cocoa butter, which occurred in 75 minutes in the solvent-free system at 60°C; this corresponds to 0.77%/hr and mg Lipozyme. Stearoyl-dioleoyl glycerol (StOO) is included in the trisaturated glycerides, as the HPLC method used was unable to separate this peak from tripalmitate.**

*Support screening.* Five supports were chosen for further investigation of the interesterification activity of the four powder CLP's which had the highest activity on the palm oil substrate. In order to obtain a simple measure of the reaction rate on different supports, a simpler substrate mixture was used in this experimental series. A substrate consisting of triolein and palmitate (in the form of either palmitic acid, methyl palmitate, or ethyl palmitate) was used (molar ratio 1:3). The reactions were conducted in heptane at 40°C to eliminate possible differences in the thermal stability of enzyme/support systems inherent in the high temperatures required for solvent-free interesterification and to dissolve the

**TABLE 3**

**Rates of Interesterification on Five Supports**

| Commercial lipase preparation | F4 | F3 | Röhm 2212E | Gist-brocades |
|---|---|---|---|---|
| | Reaction rate [$\Delta\pi$/wt protein (mg) × time (h)] × 1000 | | | |
| Substrate: Palmitic acid | | | | |
| Polyethylene | 0.04 | 0.43 | 2.7 | 3.1 |
| Celite | 0.65 | 2.2 | 2.7 | 3.1 |
| Hyflo supercel | 0.90 | 3.7 | 4.4 | 5.1 |
| Polyamide | 0.49 | 5.4 | 2.2 | 6.0 |
| Anion exchange resin | 0.97 | 7.2 | 2.2 | 9.9 |
| Substrate: Ethyl palmitate | | | | |
| Polyethylene | 3.7 | 0.16 | 7.0 | 8.4 |
| Celite | 2.5 | 4.2 | 6.3 | 8.2 |
| Hyflo supercel | 3.4 | 3.0 | 4.6 | 8.0 |
| Polyamide | 2.1 | 0.30 | 5.8 | 1.4 |
| Anion exchange resin | 0.57 | 6.3 | 1.2 | 9.0 |

Reaction: Interesterification of triolein (0.6 mM) and palmitic acid or ethyl palmitate (1.74 mM). Commercial lipase preparations (10 mg) were dissolved in 100 μL 10 mM phosphate buffer, pH 7 and immobilized on 90 mg support; 5 μL water and 2.3 g (3.5 mL) water-saturated n-heptane added. The reaction was carried out at 40°C with reciprocal shaking at 150 RPM. Rate expression is calculated as the percent incorporation of palmitate into triglycerides after 18 hr. The rate expression is: Rate = [$\Delta\pi$/wt protein (mg) × time (h)] × 1000.

palmitic acid. The rate was calculated after 18 hr by measuring the incorporation of palmitate into triolein (Table 3). The incorporation of palmitate into triolein is expressed by the variable $\pi$, which is analogous to $\Sigma$ in the stearate system: $\pi$ = (%POO + 2[%POP])/2(%OOO + %POO + %POP) × 100.

Constant $\pi$ values were obtained after 40–150 hr and no tripalmitate was detected prior to this. Some general patterns can be noted with palmitic acid as substrate. CLP's immobilized on anion exchange resin produced the highest reaction rate in three out of four cases. It is possible that there are differences in the distribution of the substrate depending on the nature of the support. Anion exchange resin, with its positive charges, would tend to attract carboxylic acids, leading to a higher concentration of substrate in the proximity of the enzyme. CLP's expressed low or intermediate activity immobilized on polyethylene supports. Hyflo supercel gave uniformly higher rates than celite and polyethylene supports, and was clearly the best support for Röhm 2212E; others report higher interesterification activity for an *Aspergillus* lipase immobilized on Hyflo supercel than on celite in the reaction of palm oil mid fraction with myristic acid as acyl donor (20). The variations of activity between CLP's immobilized on the polyamide support were similar to those observed when they were immobilized on anion exchange resin. There are large differences between the activity of Enzymatix F3 and Gist-brocades *R. arrhizus* enzyme preparations immobilized on different supports, and may reflect a greater sensitivity of these enzymes to the support characteristics. The activity of all Enzymatix F4 preparations with the acid substrate is very low.

A different pattern emerged when the ethyl ester was

used. Two CLP's had the highest rate when immobilized on the anion exchange resin. Polyethylene was a good support for three of the lipase preparations; Röhm 2212E and Enzymatix F4 displayed maximal activity on this support. Hyflo supercel is not a better support than celite with this substrate. Polyamide gave preparations with low activity in three of four cases. Three CLP's displayed large variations in activity depending on the support used; the Gist-brocades CLP expressed uniformly high activity on all supports except polyamide. Enzymatix F4 was affected by the choice of substrate, and was more active with the ethyl ester substrate than with palmitic acid. Differences in the rates here may reflect differences in the sensitivity of the different lipases to the ethanol generated in the enzymatic reaction and differences in the ethanol distribution. The ethanol could be attracted to the support or the enzyme surface, leading to the effects described above. Methyl palmitate was also tested; it produced rates which were similar to but slightly lower than tests with ethyl palmitate.

None of the enzymes tested had uniformly high activity independent of support with substrate either as the ethyl ester or the acid. It is evident that in order to obtain high reaction rates, enzyme, support and substrate must be matched so that a suitable combination is found.

## ACKNOWLEDGMENT

The support of Karlshamns AB, Karlshamn, Sweden is gratefully acknowledged.

## REFERENCES

1. Macrae, A.R., *J. Am. Oil Chem. Soc. 60*:243A (1983).
2. Macrae, A.R., in *Biocatalysts in Organic Synthesis,* Elsevier Science Publishers B.V., Amsterdam, Netherlands, 1985, pp. 195–208.
3. Kilara, A., *Process Biochemistry, April*:35 (1985).
4. James, A.T., *J. Am. Oil Chem. Soc. 62*:204 (1985).
5. Iwai, M., and Y. Tsujisaka, in *Lipases* (B. Borgström and H. Brockman, eds.), Elsevier, Amsterdam, 1984, pp. 443–469.
6. Tsujasaki, Y., S. Okumuru and M. Iwai, *Biocheim. Biophys. Acta 489*:415 (1977).
7. Reslow, M., P. Adlercreutz and B. Mattiasson, *Appl. Microbiol. Biotechnol. 26*:1 (1987).
8. Reslow, M., P. Adlercreutz and B. Mattiasson, *Eur. J. Biochem. 172*:573 (1988).
9. Theimer, R., and I. Rosnitschek, *Planta 139*:249 (1978).
10. Goderis, H.L., G. Ampe, M.P. Feyten, B.L. Fouwé, W.M. Guffens, S.M. Van Cauwenbergh and P.P. Tobback, *Biotechnol. Bioeng. 30*:258 (1987).
11. Podlaha, O., and B. Töregaard, *J. High Resolut. Chromatogr. Chromatogr. Commun. 5*:553 (1982).
12. Layne, E., *Methods Enzymol. 3*:447 (1957).
13. Graille, J., J. Muderhwa and M. Pina, *Fat Sci. Technol. 89*:224 (1987).
14. Kawase, M., and A. Tanaka, *Enzyme Microb. Technol. 11*:44 (1989).
15. Macrae, A.R., in *Microbial Enzymes and Biotechnology* (W.M. Fogart, ed.) Applied Science Publishers, 1983, pp. 225–250.
16. Moore, H., A.W. Brench and A.R. Macrae, *Eur. Pat. Appl.* EP 0199580 A2, Oct. 29, 1986.
17. Lilly, M.D., and P. Dunnill, *Ann. N.Y. Acad. Sci. 501*:113 (1987).
18. Wisdom, R.A., P. Dunnill and M.D. Lilly, *Enzyme Microb. Technol. 7*:567 (1985).
19. Uçar, T., H. Ekiz, S. Celeb and A. Caglor, in *Biocatalysis in Organic Media* (C. Laane, J. Tramper and M.D. Lilly, eds.), Elsevier, Amsterdam, 1986, pp. 381–386.
20. Wisdom, R.A., P. Dunnill and M.D. Lilly, *Enzyme Microb. Technol. 6*:443 (1984).

[Received October 30, 1989; accepted March 30, 1990]