Substrate Preferences for Lipase-Mediated Acyl-Exchange Reactions with Butteroil Are Concentration-Dependent

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Substrate preferences for pancreatic lipase-mediated acyl-exchange reactions with butteroil were concentration-dependent for the series of acyl donors and alcohol acceptors evaluated. For acidolysis reactions, the initial reaction rates and percent reaction yields after 18 h at 50 μmol acyl donor per gram substrate mixture were similar for *n*-fatty acids and their methyl and glycerol esters. At 400–500 μmol g^{-1} (and greater), order of initial reaction rates and percent reaction yield was fatty acid glycerol esters > fatty acid methyl esters > fatty acids. At concentrations above 300–500 μmol g^{-1}, reaction inhibition was observed for fatty acid substrates, and inhibition took place at lower concentrations for the shorter-chainlength fatty acids of those evaluated (5–17 carbons). Inhibition was primarily attributed to acidification of the microaqueous environment of the lipase. Desorption of water by the fatty acid substrate may be a secondary mode of inhibition. The concentration dependence of initial reaction rates and percent reaction yield was similar for the *n*-alcohol substrates evaluated (2–15 carbons) for alcoholysis reactions with butteroil. Optimum alcohol concentration was 375–500 μmol g^{-1} (except for butanol, which was 1 mmol g^{-1}), above which reaction inhibition was observed. Inhibition was attributed to desorption of water from the enzyme by the alcohol substrate. Relative reactivity of classes of alcohols for this reaction system was primary alcohols > secondary alcohols > tertiary alcohols. Generally, alcoholysis reactions were faster than acidolysis reactions, and triacylglycerols were the best substrates for acidolysis reactions with butteroil at high levels (up to 2 mmol g^{-1}) of acyl donor substrate.

KEY WORDS: Acidolysis, acyl exchange, alcoholysis, anhydrous, butteroil, lipase, modification, substrate preferences.

Butteroil ranks third in global production of edible fats and oils (1). Growing surpluses of butteroil, as well as of some other edible oils, can be expected in the future because of increasing consumer demand for foods low in fat, saturated fats and cholesterol (2). The need to obviate the impending problem of surplus oils and the advent of new technology have led to a resurgence of research on chemical and enzymic processes for modifying food lipids (3–5). Many of these recent efforts have evaluated novel reaction configurations, such as reverse micelles or microemulsions (6–8) or the use of organic solvents (9,10) to mediate lipid transformations. Other studies (11,12) have demonstrated that the lipids to be modified can also serve as a continuous phase, as well as substrate, for the reaction. In such cases, this renders the use of solvents of questionable safety unnecessary for modification processes.

Butteroil is a good substrate for evaluating reaction requirements of modification processes intended to derive specific end-products from triacylglycerols. This is because butteroil is expected to become in increasing surplus (2), and the complexity of butteroil in terms of its fatty acid composition and distribution (13) render it a particularly difficult material to transform into specific end-products. Although it is more expensive than other edible oils, butteroil has a reservoir of fatty acids that can impart desirable flavor notes (14). Thus, unlike other food lipids, it may be possible to modify butteroil into derivatives that have the attendant functionality, with flavoring potential remaining a secondary functional property.

To develop the means to specifically modify butteroil into potentially useful and functional ingredients, an understanding of the reaction requirements of the selected modification procedure is required. Our initial studies have focused on identifying optimal conditions for lipase-mediated acyl-exchange reactions with butteroil and *sn*-1,3-specific lipases in the absence of organic solvent (15). The rates of acidolysis reactions with butteroil were similar for fatty acids and fatty acid glycerol esters as acyl donors when used at low (up to 300 mM) concentrations. However, at concentrations approaching 2 M, fatty acid glycerol esters were much better than fatty acids as substrates for this reaction. Thus, under conditions where a substantial degree of product yield is expected, choice of acyl donor substrate could be critical to the design of an effective acyl-exchange modification process. The focus of the present work was to evaluate the substrate preferences for lipase-mediated acidolysis and alcoholysis acyl-exchange reactions with butteroil as a function of concentration for a number of acyl donors and alcohol acceptors.

EXPERIMENTAL PROCEDURES

Materials. Anhydrous butteroil, specified to be ≤0.15% water, was obtained from Level Valley Dairy (West Bend, WI) and stored at 2–4°C. Prior to use, the butteroil was melted at 50°C and dried with sodium sulfate to remove any residual water. Trioctanoylglycerol (triC8:0), fatty acids (FA), fatty acid methyl esters (FAME), fatty alcohols (FAOH) and other alcohol reagents were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents were high-performance liquid chromatography (HPLC)-grade and were obtained from Baker Chemical Co. (Phillipsburg, NJ) or Aldrich Chemical Co. (Milwaukee, WI). All other reagents were reagent-grade or better.

Lipases (triacylglycerol hydrolase, EC 3.1.1.3) used included a crude porcine pancreas preparation (type II, Lot #39F0454, 25% protein, 70 U mg^{-1} protein with triacetin as substrate; Sigma) and an immobilized *Mucor miehei* lipase (Lipozyme IM-20, lot #1214, 25 interesterification U g^{-1} based on incorporation of palmitic acid into olive oil; Novo Nordisk Bioindustrial, Inc., Danbury, CT). Water content of the enzyme preparations as obtained from the manufacturers was measured by the Karl Fischer method, and pH of a solution or suspension of each enzyme was recorded. The pancreatic lipase had a water content of 4.7% and a pH of 6.25, whereas the Lipozyme IM-20 preparation had a water content of 9.5% and a pH of 3.75. The enzymes were placed in a desiccator at 22–25°C for 1–2 d prior to use.

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Measurement of esterification activity of lipases. Activities of the two lipases were standardized on the basis of esterification activity in a mixture containing 300 μmol nonanoic acid (C9:0) in butteroil (1 g total substrate mixture) and 0.1 g of lipase at 60°C. The initial rate of C9:0 esterification to butteroil acylglycerols was about twice as fast for the Lipozyme IM-20 as compared to the pancreatic enzyme. Thus, to make comparisons between the two enzymes on an equal basis, Lipozyme IM-20 was used at one-half the level of addition of the pancreatic enzyme.

Reaction conditions. Reactive mixtures contained 0.05 g (Lipozyme IM-20) or 0.10 g (pancreatic lipase) enzyme and 1.0 g substrate mixture. Substrates were dissolved in butteroil and, due to subtle differences in volumes of the substrate mixtures, concentrations of substrates are presented in terms of μmol g^{-1}. Reactive mixtures were subject to magnetic stirring in closed vials and incubated at 60°C in water-jacketed vessels. Reactions were initiated by the addition of enzyme and quenched by passing the sample through a 0.2 μm nylon filter, removing particulate enzyme. Ten volumes of hexane containing undecanoic acid (C11:0), undecanoic acid methyl ester (C11:0ME) or linalool (1.6 g L^{-1}) as internal standard were added to the sample. Initial velocities of reactive mixtures were determined from samples taken within the first 30 min of incubation, where linear rates were confirmed by preliminary studies. Periodic analysis of substrate mixtures in the absence of enzyme indicated that nonenzymic reactivity was limited to less than 2% of the values reported in this paper.

Lipid analysis. Reaction rates and yields were determined on the basis of the disappearance of specific substrates (*i.e.,* FA, FAOH and triC8:0) or the appearance of specific products (esterified FA). FA were determined as their *p*-bromophenacyl ester derivatives by HPLC with a reverse-phase column at 10°C, as previously described (16). To follow the esterification of FA substrates to butteroil acylglycerols (AG), esterified FA were analyzed after conversion to their FAME derivatives by reaction with 0.5 N KOH in anhydrous methanol for 15 min at 55°C. FAME and FAOH were determined by gas-liquid chromatography (GLC) with a 10% SP-2330, 100/200 mesh, Chromosorb WAW glass column (Supelco Inc., Bellefonte, PA) and flame detection. Injector and detector temperatures were 270 and 300°C, respectively, and the column temperature was ramped from 70 to 220°C at 6°C min^{-1}. TriC8:0 analysis was done by HPLC with a reverse-phase column at 22–25°C and a light scattering detector as previously described (15). TriC8:0 was used as a model substrate for triacylglycerols (TAG) because it could be easily resolved from the other AG products in the reactive mixtures by the HPLC method used. Quantitation of products or reactants was done relative to external and internal standards.

Because the reactivity of pancreatic lipase is *sn*-1,3-specific (17), each mole of triC8:0 was considered to have two moles of reactive acyl groups. In addition, as the reactivity of triC8:0 was followed by its disappearance on an HPLC chromatogram, determinations of reactivity of acyl groups of triC8:0 are not exact. Initial rates of octanoyl group exchange from triC8:0 are underestimated because loss of only one of two reactive octanoyl groups led to a disappearance of triC8:0 on the HPLC chromatogram, even though both of the octanoyl groups could have been exchanged. An estimate of reactivity of acyl groups of triC8:0 based on probability, and assuming both primary octanoyl groups are of equal reactivity, is $\chi + \chi^2$, where χ equals the mole fraction of triC8:0 disappeared. Offsetting this underestimation is the observation that 10–20% of the disappearance of triC8:0 during the initial rate period in the reactive mixtures could be attributed to hydrolysis. In terms of the magnitude of effect, these factors roughly balance each other, making our estimates of initial rates of acyl exchange reactions for octanoyl groups of triC8:0 reasonably accurate for the purposes of the comparisons made in this paper. When results are discussed for analyses of reaction mixtures incubated for periods beyond the initial rate period, the effect of hydrolysis becomes of progressively decreasing importance because the accumulation of FAs to near steady-state levels takes place within the first 60 min under the conditions studied, whereas acyl exchange processes continue (15).

RESULTS AND DISCUSSION

Effect of enzyme concentration. The level of porcine pancreatic lipase added was varied from 0.025 to 0.40 g enzyme per gram substrate mixture, using 300 mM free nonanoic acid (C9:0) as acyl donor to butteroil AG (Fig. 1). This concentration of free C9:0 was expected to yield near maximal rates based on our previous work (Ref. 15; also see Fig. 2). Initial reaction rates were linear up to a mass ratio of enzyme/substrate of at least 0.1:1 (Fig. 1). At ratios above 1.5:1, further increases in reaction rate clearly were not linearly proportional to added enzyme. Therefore, a ratio of pancreatic enzyme/substrate of 0.1:1 was chosen to maximize efficiency of the biocatalyst.

Concentration effect of FA substrates. In a previous study, we observed inhibition of acyl-exchange (acidolysis) reactions with butteroil at levels of free undecanoic acid (C11:0) above 250 mM (15). To evaluate this phenomenon further, we studied the effect of chainlength of FA donor substrates on the concentration dependence of initial rates of acyl-exchange reactions with butteroil (Fig. 2). As acyl chainlength increased from pentanoic acid (C5:0) to heptadecanoic acid (C17:0), an increase in the optimal concentration of FA from about 150 to 500 μmol g^{-1} was observed. Generally, greater initial reaction rates and lesser degrees of inhibition were observed for the longer-chain (C13:0 and C17:0) as compared to the shorter-chain (C5:0 and C9:0) free acids and concentrations evaluated.

The acyl chainlength dependence suggests a partitioning effect of the FA substrate on reaction rates. Because of the chainlength dependence of FA solubilities in water (18), a greater mole fraction of added FA substrate would be expected to occupy the enzyme/butteroil interface or be partitioned into the microaqueous phase surrounding the enzyme for the shorter-chain than for the longer-chain FA. The inhibitory nature of the FA substrate could be attributable to either desorption of essential water from the enzyme by the polar moiety of the acid or acidification of the aqueous microenvironment of the enzyme. Both of these effects would be expected to be concentration-dependent. Increases in acyl chainlength of n-FAs from 5 to 9 decrease the proton dissociation constant from 1.56×10^{-5} to 1.26×10^{-5}, and further increases in acyl chainlength from 9 to 14 have negligible effects on the

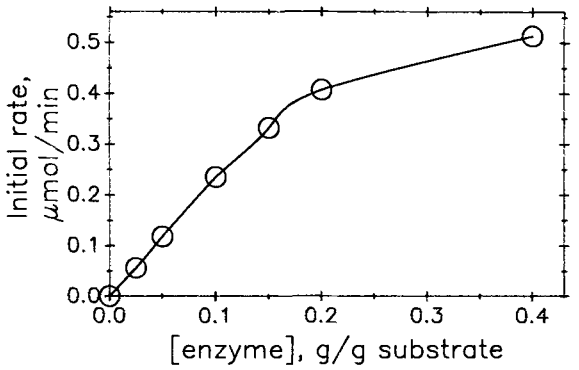

FIG. 1. The effect of enzyme concentration on initial rates of acyl exchange between nonanoic acid and butteroil acylglycerols. The substrate mixture contained 300 μmol nonanoic acid in butteroil. Reactive mixtures contained 1 g substrate mixture and varying amounts of pancreatic lipase. Reaction temperature was 60°C, and the initial rates of nonanoic acid esterification to butteroil acylglycerols are reported. Results are from two experiments where the coefficient of variation was less than 5%.

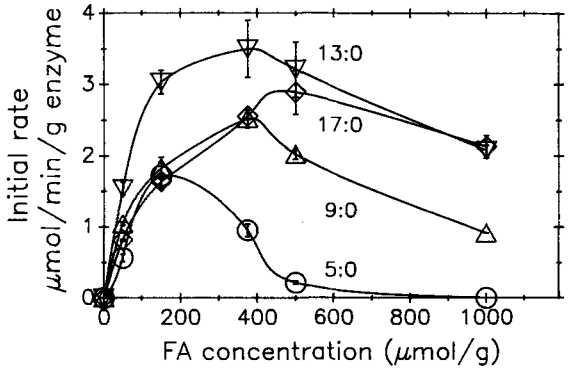

FIG. 2. Effect of fatty acid (FA) concentration on initial rates of acyl exchange with butteroil acylglycerols. Reactive mixtures contained 0.1 g pancreatic lipase and 1 g substrate (FA plus butteroil) and were incubated at 60°C. Mean initial rates of esterification of fatty acid (pentanoic acid, 5:0; nonanoic acid, 9:0; tridecanoic acid, 13:0; heptadecanoic acid, 17:0) to butteroil acylglycerols are reported, where error bars represent ±SD for 3-4 experiments for each FA.

dissociation constant (18). Thus, if acidification of the enzyme is the cause of the observed inhibition, the differences in reactivity of the FA examined (Fig. 2) are probably not due to differences in acidity. A chainlength dependence of lipase-mediated n-FA esterification with 1-hexadecanol in hexane was observed by Nishio and Kamimura (19). They found that at 1 M acyl group, n-FAs of 8–12 carbon lengths were of nearly equal reactivity, and reactivity progressively declined by about 30, 50 and 70% as acyl chainlength was decreased to 6, 4 and 2 carbons, respectively. Inhibition was attributed to acidification of the microaqueous medium by the short-chain acids, and this chainlength dependency is qualitatively similar to what we observed. Recent work has verified that enzyme pH can change in microaqueous reaction systems where consumption or liberation of acidic substrates takes place (20).

It is also possible that interfacial partitioning of FA substrates and attendant obstruction of the interface could hinder access of acyl acceptors, butteroil AG, to the enzyme and reduce reaction rates. This mechanism of inhibition would also be expected to be dependent on relative amphiphilicity of the FA substrates, which is chainlength dependent for n-FA. One mechanism of inhibition that can probably be discounted is that based on viscosity. As FA concentration was increased, the proportion of butteroil oil decreased in the reactive mixture. At 60°C, for the substrates of acyl chainlengths evaluated, the viscosity of the FA used is less than that of monoacid TAG of C10:0 or greater (21). Thus, greater reaction rates, based on decreased viscosity and attendant increases in mass diffusion rates with increasing FA might be expected to take place. One exception is free C17:0, the viscosity of which may be greater than the TAG species present in butteroil. In addition, because the melting point of free C17:0 (as the C′ form) is 61–62°C (21), some of this substrate may be in solid form in the reaction mixtures. This may account for the somewhat anomalous behavior of C17:0 observed relative to the other FAs evaluated (Fig. 2).

To determine if the inhibitory effect of FA was related to desorption of water from the enzyme, 0.045–0.9% (w/w) water was added to a reactive mixture containing 100–500 μmol C9:0 FA g^{-1} (Fig. 3). The initial rate of exchange between C9:0 and butteroil AG was at maximum values of about 1.5 μmol min^{-1} g $enzyme^{-1}$ at 100 μmol C9:0 g^{-1} at 0–0.2% added water, 3.1–3.8 μmol min^{-1} g $enzyme^{-1}$ at 250 μmol C9:0 g^{-1} at 0.045–0.45% added water, and 4.7 μmol min^{-1} g $enzyme^{-1}$ at 500 μmol C9:0 g^{-1} at 0.45% added water. That no rate enhancement of esterification was observed when water was added to the reactive mixture containing 100 μmol C9:0 g^{-1} indicated that, at this level of C9:0, sufficient water was originally available to fully support acyl-exchange reactions. These results indicate that desorption of critical water from the enzyme by elevated levels of FA substrate may be a mechanism that contributed to the reaction inhibition observed in Figure 2. At 0.45–0.9% added water, esterification activity declined at all levels of C9:0 evaluated. This was associated with FA accumulating to levels of 300 μmol g^{-1} in the reactive mixtures after 3-h incubation, indicating a shift in reaction equilibria toward net hydrolysis and away from esterification. As added water content was increased, a progressive increase in caking of the enzyme was noted; this also could have contributed to the decline in activity at the greater added water contents.

Effect of nature of substrate acyl group. The effect of acyl donor group of initial rates of acyl exchange reactions with butteroil AG was evaluated to determine the role that the chemical nature of the carboxyl group had in terms of reactivity and reaction inhibition (Fig. 4). The concentration dependence of pancreatic lipase-mediated acyl-exchange (acidolysis) reactions was compared for free undecanoic acid (C11:0), methyl undecanoate (C11:0ME) and trioctanoylglycerol (triC8:0). Only free C11:0 showed substrate inhibition at levels above 250 μmol g^{-1}. In contrast, increasing reaction rates were observed for increasing concentrations up to 1760 μmol reactive acyl group g^{-1} for C11:0ME and triC8:0. These results further suggest that the mechanism by which FA exhibits substrate inhibition is by acidification of the enzyme microenvironment. Interestingly, reaction inhibition at high FA levels was not observed for Lipozyme IM-20, as compared to the

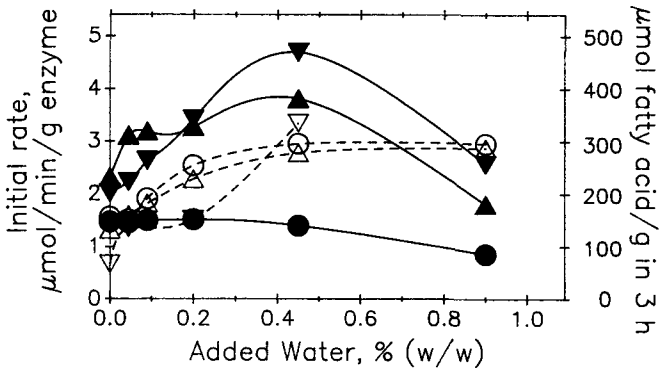

FIG. 3. Effect of added water content on initial rates of acyl exchange between fatty acids and butteroil acylglycerols and the levels of fatty acid accumulation. Reactive mixtures contained 0.1 g pancreatic lipase and 1 g substrate (100–500 μmol nonanoic acid plus butteroil) and varying levels of added water. Mixtures were incubated at 60°C. Mean initial rates of esterification of nonanoic acid (at 100 μmol/g, ●; 250 μmol/g, ▲, and 500 μmol/g, ▼) to butteroil acylglycerols are reported. Accumulation of fatty acids (not including nonanoic acid) after 3 h reaction are reported at nonanoic acid levels of 100 μmol/g, ○; 250 μmol/g, △; and 500 μmol/g, ▽. Results are representative of two experiments.

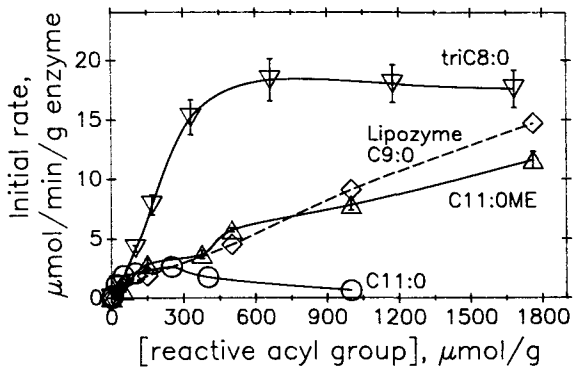

FIG. 4. Effect of reactive acyl group acid concentration on initial rates of acyl exchange with butteroil acylglycerols. Reactive mixtures that contained undecanoic acid (C11:0), methyl undecanoate (C11:0ME), and trioctanoylglycerol (triC8:0) also contained 0.1 g pancreatic lipase. Reactive mixture that contained nonanoic acid (9:0) contained 0.05 g Lipozyme. All mixtures contained 1 g substrate mixture (fatty acyl component plus butteroil) and were incubated at 60°C. Mean initial rates of the loss of triC8:0 or the esterification of fatty acid groups of C11:0 and C11:0ME to butteroil acylglycerols are reported, where error bars represent ±SD for 3–4 experiments for each fatty acyl substrate. The results with Lipozyme were from two experiments where the coefficient of variation was less than 5%.

pancreatic enzyme, indicating an enzyme-specific property of FA inhibition.

Others have noted inhibition of esterification reactions at concentrations of butyric acid above 0.5 M (22) and 0.2 M (23). Although it was not evaluated, the conclusion of those investigators was that reaction inhibition by butyric acid was caused by acidification of the microenvironment of the enzyme.

Figure 4 also reveals information on the concentration dependence of substrate preferences for acyl-exchange reactions with butteroil AG. At 50 μmol reactive acyl group g^{-1}, the ratios of initial reaction rates with free C11:0, C11:0ME and triC8:0 were about 3.1:1:2.6. At 400 μmol reactive acyl group g^{-1}, relative reaction rates were about 1:2.5:9.3 for free C11:0, C11:0ME and triC8:0. We do not expect that the difference in acyl chainlength between octanoyl and undecanoyl residues would greatly influence the general trends observed when comparing methyl and glycerol esters of fatty acids as substrates for acyl-exchange reactions with butteroil (see also next paragraph). There have been few other reports of acyl group preference for lipase-mediated acyl-exchange reactions, and none that we could find that evaluated the concentration dependence of these preferences. Schuch and Mukherjee (24) found that at 100 mM, relative rates of acyl exchange for the acyl donors evaluated were heptadecanoic acid > triolein > methyl heptadecanoate. Using their initial data point from the published progress curves, we calculated the relative (initial) reaction rates to be 1.8:1.5:1. Our results are similar in that at relatively low levels of reactive acyl groups, free acids and glycerol esters are more reactive as acyl donors than methyl esters. Using immobilized lipases and low levels of substrates (up to 3 mM) dissolved in heptane, greater reaction rates were reported for exchange between ethyl palmitate and triolein, as compared to palmitic acid and triolein (25). However, this preference for substrate appeared to be dependent upon the support matrix used for immobilization. In a subsequent study with the same substrates (at high concentrations) in the absence of solvent, greater rates of acyl exchange were observed with ethyl palmitate than with palmitic acid when Lipozyme was used as the catalyst (26).

The effect of FAME acyl chainlength was evaluated (Fig. 5). A similar dependency of initial reaction rates on concentration for methyl heptanoate (C7:0ME), methyl tridecanoate (C13:0ME) and methyl heptadecanoate (C17:0ME) was observed at levels up to 1760 μmol g^{-1}. A slight preference for the shorter-chain FAME within the series evaluated, appeared to exist for acyl-exchange reactions with butteroil. A qualitatively similar but more pronounced preference for methyl esters of shorter-chainlength n-FAs (tested only at 100 mM) was reported for lipase-mediated acyl-exchange reactions with TAG by Schuch and Mukherjee (24). The concentration dependence of reactivity of FAMEs is in marked contrast to those obtained for the FA series of substrates (Fig. 2). The lack of substrate inhibition of FAME compared to FA substrates is probably due to the lesser polarity and lesser tendency to partition at the interface of the former. In addition, FAME substrates have no ionizable carboxyl group to acidify the enzyme microenvironment.

We further evaluated the inhibitory nature of high levels of FA (1760 μmol g^{-1} each of tetradecanoic acid and 1-decanol) by comparing the time course of ester synthesis reactions mediated by Lipozyme IM-20 and pancreatic lipase (Fig. 6). Ester synthesis was rapid for the Lipozyme IM-20 preparation, and a 70% yield of decanoyl tetradecanoate was observed after only 2 h. In contrast, a 2-h lag period was noted for the pancreatic lipase, and a 70% reaction yield was observed after 7.5 h. One reason for the difference in activity between these catalysts is that the water content of the Lipozyme IM-20 was about twice that of the pancreatic enzyme. A limitation in water for the latter enzyme, especially at the levels of decanoic acid and tetradecanol used, may account for the initial lag period and the autocatalytic effect whereby progressive

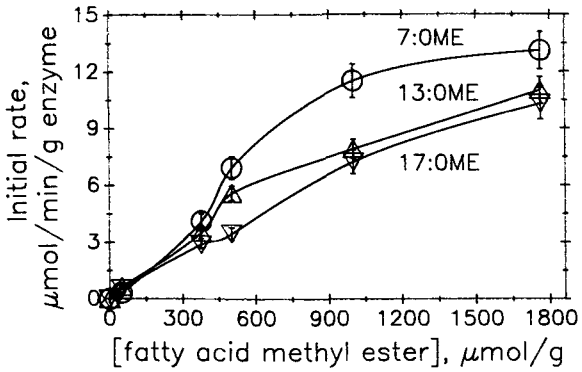

FIG. 5. Effect of fatty acid methyl ester acid concentration on initial rates of acyl exchange with butteroil acylglycerols. Reactive mixtures contained 0.1 g pancreatic lipase and 1 g substrate (fatty acid methyl ester plus butteroil) and were incubated at 60°C. Mean initial rates of esterification of methyl heptanoate (7:0ME), methyl tridecanoate (13:0ME) and methyl heptadecanoate (17:0ME) to butteroil acylglycerols are reported, where error bars represent ±SD for 3–4 experiments for each substrate.

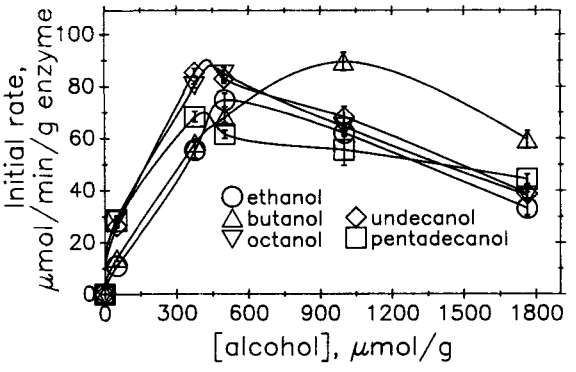

FIG. 7. Effect of alcohol concentration on initial rates of acyl exchange with butteroil acylglycerols. Reactive mixtures contained 0.1 g pancreatic lipase and 1 g substrate (alcohol plus butteroil) and were incubated at 60°C. Mean initial rates of loss of free alcohol are reported, where error bars represent ±SD for 3–4 experiments for each alcohol substrate.

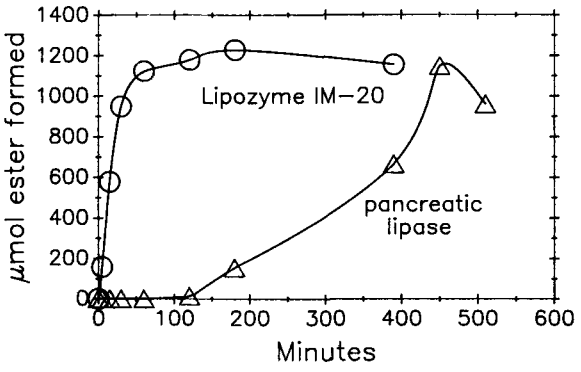

FIG. 6. Effect of enzyme source on progress curves for ester synthesis. Reactive mixtures contained 1760 μmol each of 1-decanol and tetradecanoic acid, and either 0.1 g pancreatic lipase or 0.05 g Lipozyme. Mixtures were incubated at 60°C, and the results are from two experiments where the coefficient of variation was less than 5%.

ester synthesis would yield water to support the reaction. We tested this by evaluating this reaction with the pancreatic enzyme where water was initially added to the reactive mixture at a level equivalent (0.64%) to the calculated yield of water arising from ester synthesis after 3 h for the progress curve presented in Figure 6. A progress curve almost identical to the one for pancreatic lipase appearing in Figure 6 was obtained, indicating that the lag period was not caused by a lack of water availability for the enzyme. The alternative possibility of the lag period being caused by enzyme acidification at high levels of free C10:0 was also assessed by recovering enzyme at different stages of the reaction and measuring pH of an enzyme solution or suspension. After 2 and 7.5 h of reaction, the pH of the pancreatic enzyme was 4.5 and 5.5, respectively, compared to an initial pH of 6.25 of the enzyme reagent. In contrast, the pH of Lipozyme IM-20 after 2 and 7.5 h was 3.75 and 3.55, respectively, compared to a pH of 3.75 for the enzyme as obtained from the manufacturer. Thus, from these studies (Figs. 2–4 and 6) we conclude that the primary mechanism of reaction inhibition by free FA substrates is the acidification of the enzyme, with desorption of water from the enzyme being a secondary but contributing factor.

Effect of alcohol acceptor substrates. Substrate preferences for alcoholysis reactions with butteroil were also investigated (Fig. 7). The series of alcohols evaluated displayed maximum initial rates of alcoholysis reactions at 375–500 μmol g^{-1}, except for *n*-butanol, where maximum rates were observed at 1000 μmol g^{-1}. All alcohols showed substrate inhibition at the greater concentrations evaluated, and there was little dependence of activity on chainlength. The inhibitory effect of the longer-chainlength alcohols (8–15 carbons) is probably caused by interfacial partitioning of the alcohols and the attendant loss of interfacial access by the butteroil AG. The inhibitory effect of *n*-alcohols (10–16 carbons) on lipase hydrolysis of TAG in aqueous systems has been attributed to interfacial partitioning (27). Because butanol and ethanol are fairly miscible with water, their mechanism of reaction inhibition may be different than that affected by the longer-chainlength alcohols evaluated. In microaqueous systems, ethanol concentrations greater than 0.2 M (23) and 0.4 M (22) inhibit ester synthesis, and this effect was attributed to a dehydration of the enzyme by the alcohol (23). The somewhat anomalous behavior of butanol, although reproducible, is difficult to explain. One possible explanation is that high levels of butanol may be inhibitory by both dehydration and interfacial partitioning mechanisms, whereas the other alcohols are predominantly inhibitory by only one mechanism. In any case, the effect of butanol was qualitatively similar to the other alcohols evaluated.

Activities of various classes of alcohols were also evaluated at 500 μmol g^{-1} (Table 1). Generally, reactivity of alcohols were, in decreasing order, primary > secondary > tertiary. These observations are consistent with prior studies (28,29), and it is believed that restrictions of conformational mobility of enzymes in anhydrous media render enzymes less active toward bulky substrates (10). When glycerol was a substrate for alcoholysis reactions with butteroil, initial rates of 0.26 μmol diacylglycerol

TABLE 1

Initial Rates of Acyl Exchange Reactions Between Butteroil Acylglycerols and Various Alcohol Acceptors[a]

Alcohol substrate	Initial reaction rate (μmol min^{-1} g enzyme^{-1})
Primary alcohols	
1-butanol	53.7 ± 3.9
1-octanol	68.8 ± 2.7
1-dodecanol	72.0 ± 1.7
Secondary alcohols	
sec-butanol	17.4 ± 0.5
2-octanol	27.8 ± 2.0
Tertiary alcohols	
t-butanol	6.0 ± 0.2
linalool	0

[a]Alcohol substrates were used at 500 μmol g^{-1} in butteroil at 1 g substrate mixture per 0.1 g pancreatic lipase. Initial rates are based on the disappearance of alcohol, and values represent the means ± SD for 3-4 experiments for each alcohol substrate.

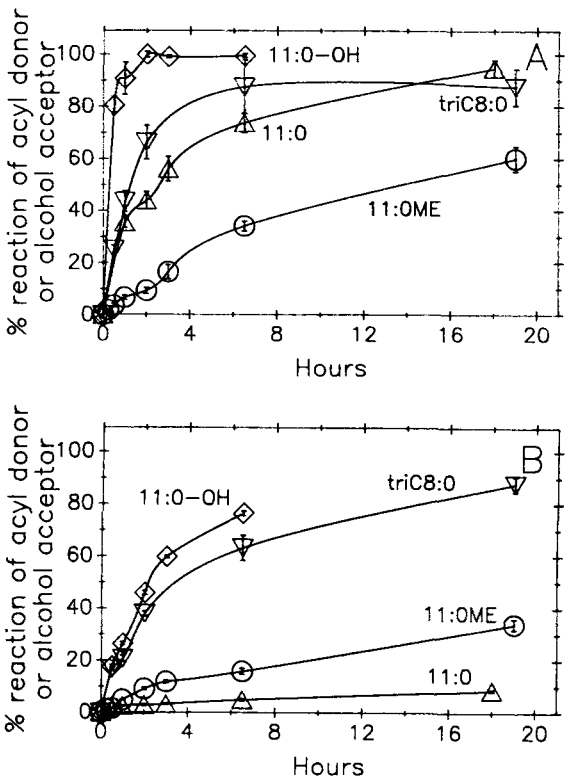

FIG. 8. Progress curves of reaction yield for various substrates for acyl exchange processes with butteroil. Reactive mixtures contained 0.1 g pancreatic lipase and 1 g substrate mixture (acyl or alcohol substrate plus butteroil) and were incubated at 60°C. Substrates included undecanol (11:0-OH), undecanoic acid (11:0), methyl undecanoate (11:0ME) and trioctanoylglycerol (triC8:0) at A, 50 μmol/g substrate mixture (equivalent to 100 μmol g^{-1} reactive acyl donor groups for triC8:0); and B, 400 (11:0) or 500 (11:0-OH and 11:0ME) μmol/g substrate mixture (triC8:0 was 331 μmol g^{-1}, equivalent to 662 μmol g^{-1} reactive acyl donor groups). The loss of 11:0-OH and triC8:0 and the esterification of acyl groups from 11:0ME and 11:0 to butteroil acylglycerols are reported as the means ±SD for 3-4 experiments for each substrate.

min^{-1} g enzyme^{-1} were observed at 125 μmol glycerol g^{-1}, with no activity observed at 333 to 1000 μmol glycerol g^{-1} (after accounting for hydrolysis). The ability of pancreatic lipase to support glycerolysis reactions was comparatively low, and this property appears to be primarily dependent on the source of enzyme (Ref. 30; and manuscript in preparation).

Effect of substrates on reaction yields. Progress curves for reactions in terms of the proportion of acyl donor or alcohol acceptor molecules reacted at 50 μmol g^{-1} (100 μmol reactive acyl groups for triC8:0 g^{-1}; Fig. 8A) and 400-500 μmol g^{-1} (331 μmol triC8:0 g^{-1} and 662 μmol reactive acyl groups g^{-1}; Fig. 8B) show the concentration dependence of reaction yields for triC8:0, free C11:0, C11:0ME and undecanol (C11:0-OH). At 50 μmol g^{-1} (Fig. 8A), nearly 100% reaction yield was observed for C11:0-OH after 2 h and about 90% reaction yield was observed for C11:0 and triC8:0 after 18-19 h. In contrast, reaction yield was limited to about 60% for C11:0ME after 19 h. At 400-500 μmol g^{-1} (Fig. 8B), reaction yield *vs.* time for C11:0-OH, C11:0ME and C11:0 decreased relative to what was observed at 50 μmol g^{-1}, whereas the reaction yield of triC8:0 *vs.* time was similar at both substrate concentrations. The decrease in reaction yield at 400-500 μmol g^{-1} for C11:0-OH and C11:0 was probably caused by reaction inhibition at these levels (Figs. 7 and 4, respectively), whereas for C11:0ME, the decrease in yield upon increasing concentration to 500 μmol g^{-1} is probably caused by the relatively slow reaction rates of this acyl donor (Fig. 4). These data indicate that TAGs are good substrates for lipase-mediated acyl exchange reactions when used at high concentrations and when a substantial degree of substrate transformation or product synthesis is desired.

Although our studies were done with butteroil as the edible oil to be modified, it is likely that our observations would qualitatively apply to enzyme-mediated acyl-exchange reactions with other edible oil sources where TAGs are the primary components.

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