Acetylacylglycerol Formation by Lipase in Microaqueous Milieu: Effects of Acetyl Group Donor and Environmental Factors

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Of six lipases evaluated, lipozyme was most efficacious at mediating acyl-exchange reactions in forming acetylacylglycerols (AcAG) from acetyl donors and olive oil triacylglycerols (TAG) in the absence of organic solvent. Reactivity among a series of 10 acetyl-donor substrates was compared for model reactions configured for acetyl transfer to 1-undecanol and ac(et)yl exchange with olive oil. Of the two acetyl donors most suitable for exchange reactions with olive oil, triacetin was a more efficient cosubstrate than was ethyl acetate. Greatest absolute yields of total AcAG were observed at molar ratios of triacetin/olive oil TAG of 1:2 and ethyl acetate/olive oil TAG of about 3:1, and respective reaction yields approached 90% and 50% of the calculated maximum for these two systems. AcAG formation was optimal at about 1–2% total water content in the system and at 80–85 °C. When organic solvents were used as an inert dispersing medium, those of intermediate polarity were most supportive of biocatalysis.

Keywords: *Lipase; acetylacylglycerols; microaqueous enzymology; acyl transfer; acyl exchange; lipid transformation; fats and oils*

INTRODUCTION

Within the past decade, attention toward using enzymes to modify lipophilic components has increased dramatically. The impetus for this is founded on a developing and fundamental understanding of enzyme action in microaqueous media (Klibanov, 1986; Dordick, 1989). Applications of this emerging technology have included exploiting the reversibility of hydrolytic enzyme reactions for facile product syntheses, preparing chiral compounds or resolving racemates, exploiting the limited plasticity of enzymes in microaqueous media to allow for enhanced thermal stability and "bioimprinting", and demonstrating new reaction specificities for enzymes relative to their activities in conventional aqueous media.

A less exotic, but perhaps more important, application of microaqueous enzymology is the transformation or "restructuring" of triacylglycerol (TAG)-rich fats and oils or their constitutive fatty acids (Macrae, 1983; Schuch and Mukherjee, 1987; Harwood, 1989; Bloomer et al., 1992), which are in vast global supplies (at least 60×10^9 kg produced annually; Jeffcoat, 1989). Although enzymes, and particularly lipases, have been shown to synthesize or modify TAG-rich lipids for decades, such processes have been developed with reaction systems employing a substantial aqueous phase (Sym, 1936; Iwai et al., 1964; Tsujisaka et al., 1977; Stevenson et al., 1979; Yokozeki et al., 1982). More recent approaches have focused on eliminating the need for a distinct aqueous phase in transforming these lipid resources into highly functional and/or value-added materials (Bell et al., 1978; Macrae, 1983; Zaks and Klibanov, 1984; Gatfield, 1986).

For substrates to be transformed into materials for human consumption, avoiding the use of solvent is desirable for the obvious reasons of safety. However, in some instances, the use of a solvent, or supercritical fluid (Nakamura, 1990), may yield tangible benefits to a biocatalytic process in terms of reaction control and/or yield (Goh et al., 1993; Yang et al., 1993a,b) or dynamic (Bruce and Daugulis, 1991) or subsequent (Carrea, 1984) product recovery.

Commercial feasibility of a biocatalytic process requires sufficient levels of substrate throughput and product yields (Bloomer et al., 1992). With particular reference to lipase-mediated lipid transformations, process design is critical, as TAG-modifying reactions can be configured as acyl-transfer or acyl-exchange reactions, with several choices of potential cosubstrates for achieving the desired transformation.

In this study, reactions were designed to transform, by acyl exchange, a TAG-rich oil into acetylated acylglycerol (AcAG) products in microaqueous media using acetate ester cosubstrates. Olive oil was used as the model TAG-rich oil because its comparatively simple TAG profile allowed resolution of TAG and AcAG products formed during reactions. AcAG products have uses as films or coatings to prevent moisture migration, antidusting and antifoaming agents, lubricants, plasticizers, and release agents (Lovegren and Feuge, 1956; Luce, 1967; Eastman Kodak, 1991). The studies reported here are also relevant to the production of the reduced-calorie lipid preparation SALATRIM, which is defined as a mixture of TAG species composed of long- and short-chain fatty acids (Smith et al., 1994).

EXPERIMENTAL PROCEDURES

Materials. Lipases (triacylglycerol acylhydrolase EC 3.1.1.3.) from porcine pancreas [type II, lot 39F-0454, 4.7% (w/w) moisture] and *Candida cylindracea* [now *C. rugosa*, type VII, lot 38F-08581, 11% (w/w) moisture] were obtained from Sigma Chemical Co. (St. Louis, MO); *Geotrichum candidum* (type GC), *Pseudomonas* spp. [type PS-30, 1.2% (w/w) moisture], and *Aspergillus niger* (type AP-12) lipases were obtained from Amano International Enzyme Co. (Troy, VA). Lipozyme IM20 [from *Rhizomucor miehei*, lot 101626, batch LM7 0504, immobilized on an ion-exchange resin, 10% (w/w) moisture] was obtained from Novo-Nordisk Bioindustrials, Inc. (Danbury, CT). These enzymes were used as supplied unless otherwise noted. Moisture contents, if indicated, were determined experimentally by Karl Fischer titration. Olive oil, pyridine,

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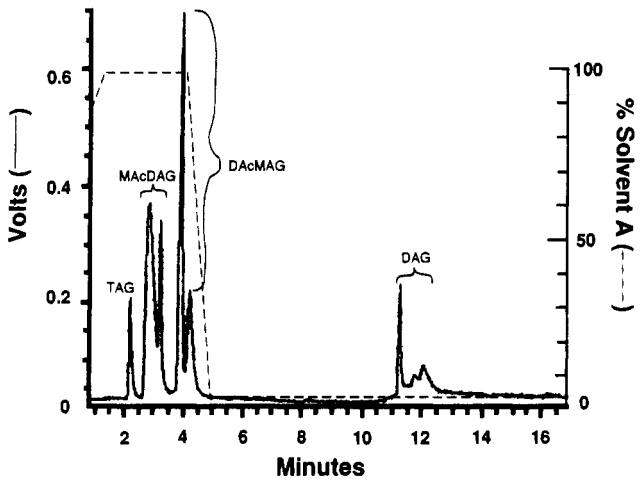


Figure 1. Liquid chromatographic separation of principal ac(et)ylglycerol components in reaction mixtures. Solvent A was hexane/chloroform/acetic acid (60:40:0.2 v/v/v), and solvent B was hexane/chloroform/acetone (5:13:7 v/v/v). Abbreviations: TAG, triacylglycerol; MAcDAG, monoacetyldiacylglycerol; DAcMAG, diacetylmonoacylglycerol; DAG, diacylglycerol.

lipid standards, acetic anhydride, triacetin, and phenyl, undecanyl, and isoamyl acetates were also obtained from Sigma. Vinyl, hexyl, ethyl, isopropenyl, and cyclohexyl acetates were obtained from Aldrich Chemical Co. (Milwaukee, WI), as were the HPLC grade solvents.

Acetylated acylglycerol standards were prepared from mono- and diacylglycerol (MAG and DAG) fractions isolated from glycerolysis reaction mixtures involving olive oil, essentially as described in Yang et al. (1993a,b, 1994a). The product mixture [containing triacylglycerol (TAG), DAG, MAG, and fatty acids] was fractionated by silica gel (32–63 μm particle size, Fisher Scientific, Chicago, IL) chromatography using 3 column volumes of chloroform/hexane (4:1 v/v) followed by 4–5 column volumes of hexane/acetone/ether (6:1:3 v/v/v) under slight pressure (0.014–0.034 MPa of N_2). Fractions were collected, and a qualitative identification and estimate of purity were afforded by analysis of fractions by thin-layer chromatography on silica gel G plates (Whatman Inc., Clifton, NJ) using the hexane/acetone/ether solvent system just described. Bands were visualized by iodine staining and compared to authentic standards for TAG, DAG, MAG, and fatty acids. Samples of purified MAG and DAG fractions were acetylated by reaction with a 30-fold weight excess of acetic anhydride in pyridine at 60 °C overnight. After evaporation of excess reagent, acetic acid, and pyridine, the acetylated MAG and DAG derivatives were dissolved in a minimum volume of chloroform.

Reaction Mixtures. Compositions of reaction mixtures are provided in the figure legends. Typically, 10% (w/w) enzyme was added to a mixture of cosubstrates to initiate reaction. After selected periods of incubation, reactivity was quenched by filtering the enzyme from the mixture with a 0.2 μm nylon filter. Previous studies have shown this step to be effective in ceasing reactivity (Kuo and Parkin, 1993; Yang et al., 1993a, 1994a). The filtered reaction mixture was dissolved in chloroform (1:5 w/v) prior to analysis.

Analysis of Acetyl-Group Transfer. Liquid chromatography was used to analyze products in quenched reaction mixtures. The equipment included two Model 510 pumps and Baseline 810 software for peak integration (Waters Associates, Milford, MA). Undecanyl acetate analysis was done by isocratic elution on a Hibar Lichrosorb RP-18 column (250 mm × 4.0 mm, 5 μm; Alltech Associates, Deerfield, IL) using acetonitrile, with detection at 210 nm (Model 484 absorbance detector, Waters Associates). Acetylated acylglycerols (AcAG) were separated on a normal phase column (Econosil, 250 mm × 4.6 mm, 5 μm; Alltech Associates), using a dual solvent system, with detection by a laser light-scattering detector (Model ELSD IIA; Varex, Rockville, MD) (Figure 1). The elution program used was virtually identical to that of Yang and Chen (1991) using hexane/chloroform/acetic acid (60:40:0.2 v/v/v, solvent A) and hexane/chloroform/acetone (5:13:7 v/v/v, solvent B), except that sometimes the flow rate was varied between 1.0 and 1.5 mL/min to allow peak resolution. Between samples, the column was re-equilibrated with 10–15 mL of 89:11 solvents A:B. Triacetin and acetic acid were transparent to detection and could not be quantified. Since acetic acid was transparent to detection, the extent of hydrolysis that took place in the reaction mixtures was estimated by DAG, rather than fatty acid, content. Quantification of undecanyl acetate and AcAG species was done relative to external standard curves. For some of the acyl-exchange reactions, a corresponding alkyl ester peak eluted at 2.4–2.5 min (not shown in Figure 1), immediately after the TAG peak. Because of differences in volatility of the various alkyl esters formed (some were partially "transparent" to detection), the alkyl ester peak could not be used for quantitative purposes. Furthermore, no alkyl ester peak was formed, or expected, with isopropenyl and vinyl acetate substrates.

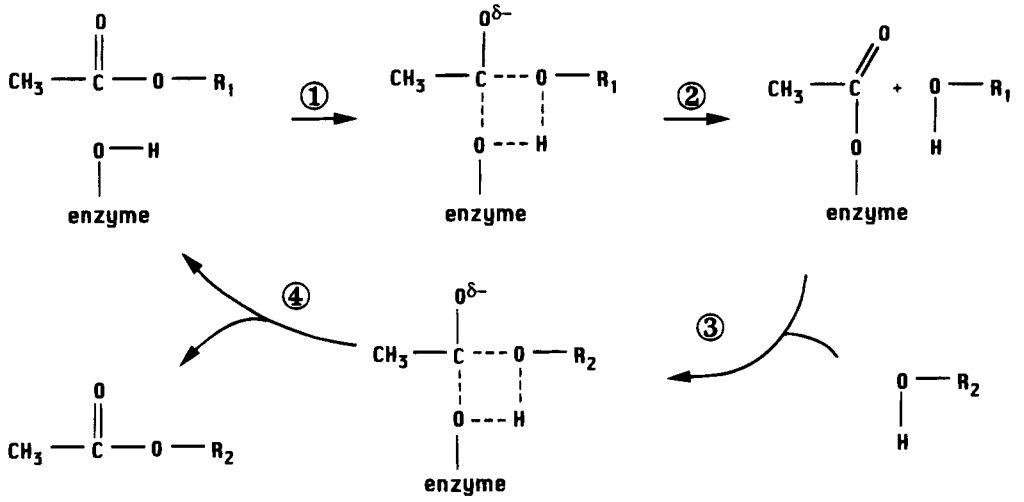
RESULTS AND DISCUSSION

Considerations Regarding Choice of Reaction Motif. Reaction mixtures for achieving ac(et)ylation of a target acceptor can be configured as esterification, acyl-transfer, or acyl-exchange processes. Our studies focused on the latter two processes, which can be delineated as unidirectional or reciprocal acyl-transfer processes, respectively. To fully understand the reactivity of the substrates selected for these reactions, it is necessary to consider the enzymic reaction mechanism most likely to exist for these processes and the subtle distinctions between them. The model mechanisms (Schemes 1 and 2) are based on the current understanding that many lipases, including that from *R. miehei*, possess the catalytic triad of Asp(Glu)-His-Ser, reminiscent of the serine proteases (Brady et al., 1990; Winkler et al., 1990; Schrag et al., 1991). Thus, the reaction mechanism of lipases equipped with this triad is believed to involve an acyl-enzyme (acyl-O-Ser) intermediate and embody electronic forces such as electrophilicity, nucleophilicity, general acid–base behavior, and charge neutralization (Carter and Wells, 1988; Brzozowski et al., 1991).

Schematics were specifically developed for acyl-transfer and acyl-exchange reactions (Schemes 1 and 2, respectively). Although these schemes are relevant to lipase-mediated reactions in general, they have been developed with the specific substrates used in the present studies in mind and for simplicity in raising the pertinent features. Competing hydrolytic reactions are not shown, although they occurred in the reaction mixtures studied to a limited degree (typically about 7% of the total ac(et)yl groups initially present). Hydrolysis would occur when water serves as a strong nucleophile to displace the ac(et)yl group from the ac(et)yl enzyme intermediate (Scheme 1, step 3; Scheme 2, steps 3 and 4).

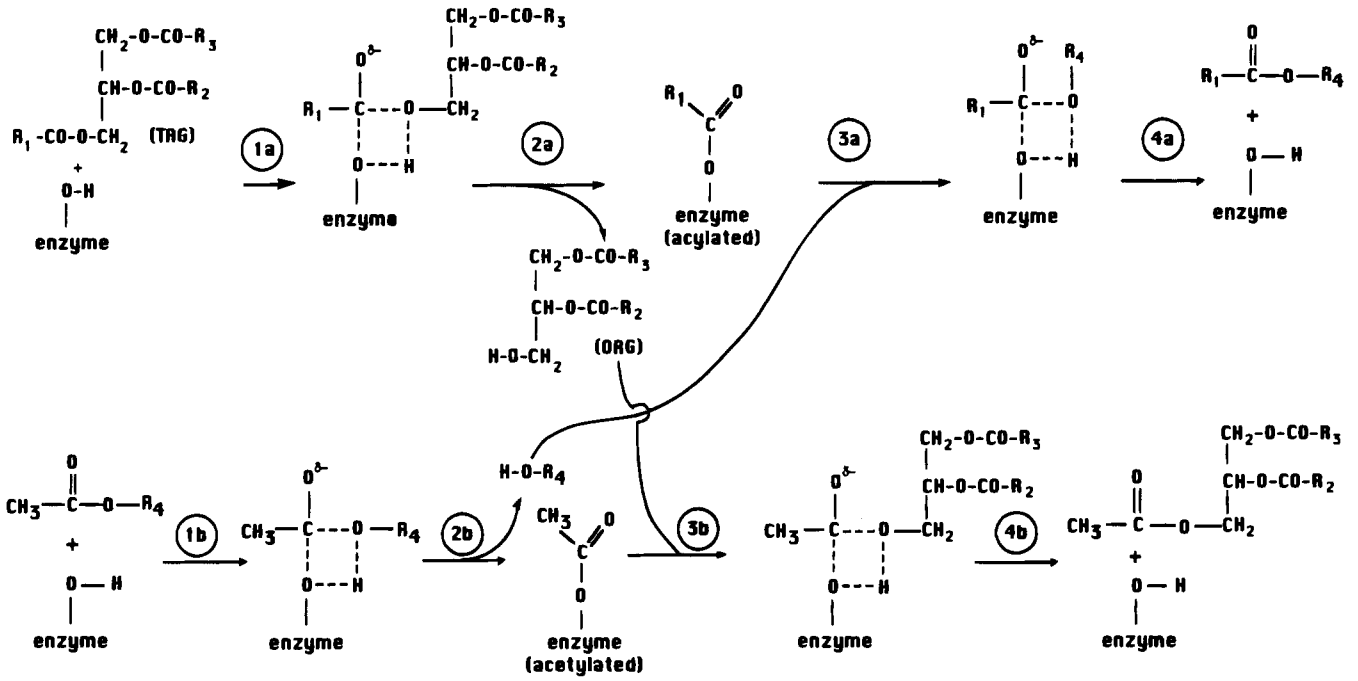
Ac(et)yl-Transfer Reactions. The most likely mechanism for ac(et)yl transfer (Scheme 1) is initiated by coordination of the acetyl donor with Ser-OH at the enzyme active site (step 1). This step should be increasingly favored by substrates with enhanced electron-withdrawal properties of the $-O-R_1$ moiety, which both enhances the electrophilicity of the carbonyl carbon atom and assists the stabilization of the developing charge (δ^-) on the carbonyl oxygen. This step should also be hindered by the presence of bulky $-O-R_1$ residues if steric constraints exist at the active site. The second step completes the process of formation of the acetyl enzyme adduct and should be increasingly favored by decreasing basicity (increasing acidity) of the

Scheme 1. Model Mechanism for Lipase-Mediated Acyl-Transfer Reaction[a]



[a] The active site serine is depicted as enzyme-OH, R_x depicts an alkyl group, and broken lines indicate bonds being broken or formed. See text for discussion.

Scheme 2. Model Mechanism for Lipase-Mediated Acyl-Exchange Reaction[a]



[a] Details same as for Scheme 1. TAG, triacylglycerol; DAG, diacylglycerol. See text for discussion.

leaving group ($HO-R_1$). In the third step, a strong nucleophile ($HO-R_2$) coordinates with the electrophilic carbonyl carbon to form another tetrahedral intermediate. Acetyl-transfer reaction is completed by the displacement of the newly formed acetate ester (step 4). With particular reference to our studies, during the initial stages of reaction, the nucleophile $HO-R_2$ is 1-undecanol, which is the common and sole acetyl group acceptor for all acetyl donors evaluated. Therefore, steps 1 and 2 in Scheme 1 are those primarily being examined by this series of substrates. Since enzyme–Ser-OH acylation (steps 1 and 2) is kinetically slower than nucleophilic displacement (steps 3 and 4) in alcoholysis reactions (Rangheard et al., 1989), the series of substrates used also evaluates the rate-limiting steps.

Ac(et)yl-Exchange Reactions. Our analysis of ac(et)-yl-exchange reactions was designed to follow the incorporation of acetyl groups into the glycerol backbone of triacylglycerols (TAG) originating from olive oil. To parallel our analytical approach, a simplified mechanistic scheme for ac(et)yl exchange is offered (Scheme 2), although it can be generally applied to other acyl-exchange reactions. For an acetate residue to become incorporated into olive oil acylglycerols (AG), an acyl group must first be removed from the original TAG to yield an acyl enzyme intermediate with a diacylglycerol (DAG) formed as a product (steps 1a and 2a). The acetyl donor substrate ($CH_3-OCO-R_4$) is likely to undergo an analogous reaction to yield an alcohol acceptor ($HO-R_4$) and an acetyl enzyme intermediate (steps 1b and 2b). Exchange is completed when $HO-R_4$ and DAG, acting as nucleophiles, coordinate and displace the ac-(et)yl group from the acyl enzyme and acetyl enzyme adducts, respectively (steps 3a and 4a and 3b and 4b). For our studies, olive oil DAG is the common and sole acetyl group acceptor for ac(et)yl-exchange reactions. Therefore, any differences in reactivity among the acetyl donor substrates evaluated can be attributed primarily to steps 1b and 2b and 3a and 4a of Scheme 2, the rates of which should increase with increasing electrophilicity

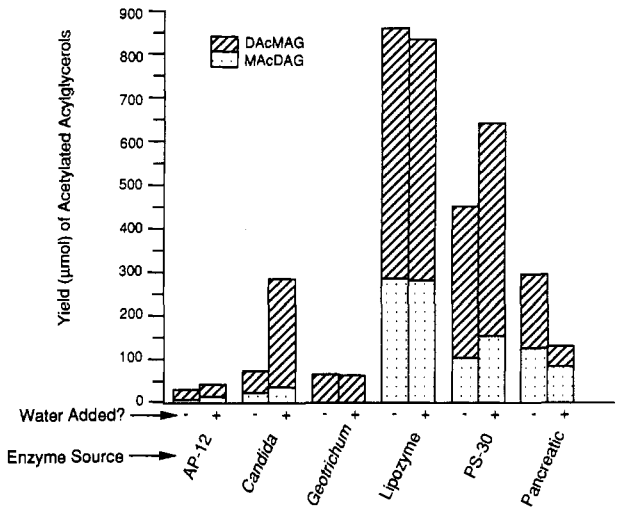


Figure 2. Screening of enzyme preparations for ac(et)yl-exchange activity. Reaction mixtures contained 0.50 g of olive oil, 0.50 g of triacetin, and 0.12 g of enzyme in the presence or absence of 10 μL of added water. Incubations were at 37 °C for 22 h. Results are from three experiments, and the average coefficient of variation was 12.6%.

of the carbonyl carbon of $CH_3-OCO-R_4$ and nucleophilicity of the acyl group acceptor $HO-R_4$. Thus, reactivity might be expected *a priori* to increase with increasing electronegativity of the O atom of the $-O-R_4$ moiety, which is enhanced by decreasing electron withdrawal by the constitutive $-R_4$ group.

One may consider that the acetate ester cosubstrate ($CH_3-OCO-R_4$) may function as a nucleophile (instead of the $HO-R_4$ adduct formed by steps 1b and 2b) to directly coordinate with the acyl enzyme intermediate and displace the acyl group as an acyl$-R_4$ ester, resulting in the acetyl enzyme intermediate. In most cases, this reaction pathway would be minimal because the nucleophilicity (and, consequently, reactivity in step 3a) of the $HO-R_4$ adduct would be greater than that of the corresponding acetate ester of R_4.

Screening of Enzymes. The ability of different lipases to produce AcAG (MAcDAG + DAcMAG) was evaluated for an acyl-exchange reaction using triacetin and olive oil (1:1 w/w) as substrates (Figure 2). On the basis of the molar ratio of acetyl/acyl groups of 4.02:1 (equivalent to a mole fractional ratio of 0.801:0.199), a completely randomized distribution of fatty acids along the glycerol backbone as a result of enzyme acyl exchange, provides for a calculated theoretical maximum yield of AcAG as 47.8 mol % [3(0.801 × 0.801 × 0.199) for DAcMAG + 3(0.801 × 0.199 × 0.199) for MAcDAG] of the total glycerol (2.85 mmol) added to the system, or 1.36 mmol of combined DAcMAG and MAcDAG. *(Assuming an sn-1,3-regioselectivity of the enzymic reaction, where the composition of the sn-2 position remains fixed at an acetyl/acyl molar ratio of 4.02:1, one arrives at the same result, making the theoretical maximum yield calculation applicable to all of the enzymes tested.)*

These lipases were compared on the basis of equal mass of catalyst preparation rather than on a normalized basis of activity (i.e., hydrolytic activity). The justification for this is that clear correlations between hydrolytic and other catalytic activities among lipases are not apparent (Vorderwülbecke et al., 1992; Yang et al., 1994a). Lipozyme IM-20 was the most effective catalyst, followed by the *Pseudomonas* sp. lipase, type PS-30, with reaction yields of 60–63% and 33–46%, respectively (Figure 2). *C. rugosa* and pancreatic lipases were less effective, allowing 5.2–21% and 17–29% theoretical maximum yield, respectively. These latter enzymes were most efficient in the absence and presence of 1% added water, respectively. The different responses to added water of these lipases are probably because of differences in both moisture content of the enzymes as supplied (Yang et al., 1993a,b) and tenacities of the lipases in binding moisture (Zaks and Klibanov, 1985). The other enzymes evaluated provided limited reaction yields (<5% theoretical maximum). On the basis of these results, lipozyme was used for most of the other studies reported here.

Acetyl Donor Preferences for Acetyl-Transfer Reactions. The extent of acetyl transfer to undecanol was determined after 3 h of incubation (Table 1). The reactivity of acetic anhydride could be accounted for entirely as nonenzymic, because in the absence of lipase a similar degree of acetyl transfer to undecanol was observed. Acetyl transfer with the other acetyl-donors was confirmed to be enzyme-mediated. Of these, vinyl acetate was the best donor, likely because the leaving group, vinyl alcohol, tautomerizes to acetaldehyde and renders the reaction irreversible (Wang et al., 1988; Santaniello et al., 1993). The relative reactivity of phenyl and benzyl acetates can be accounted for in the suitability of the phenol and benzyl alcohol leaving groups, respectively, the weakest and next weakest bases of the group of acetyl donors evaluated.

Triacetin was a moderately effective acetyl donor with undecanol, ethyl, and hexyl acetate less reactive and isoamyl and cyclohexyl acetates poor acetyl donors. These relative reactivities are what one could expect on the basis of the estimated pK_a values (Table 1) of the corresponding leaving groups. Additionally, the bulky alkyl chains for isoamyl and cyclohexyl acetates may impose additional (steric) constraints on their reactivity. Although triacetin might also be considered a "bulky" substrate, lipases have probably evolved to easily accommodate the structures of TAG relative to other substrates of similar, or even lesser, dimension.

Isopropenyl acetate was a surprisingly poor acetyl donor, considering the leaving group, 2-propenol, tautomerizes to form acetone and render the reaction irreversible (Wang et al., 1988; Santaniello et al., 1993). The lack of reactivity may be accounted for by a combination of steric and rotational hindrance, not simply because it has a secondary alcohol group. Although *R. miehei* lipase is *sn*-1,3-regioselective toward TAG substrates (Matori et al., 1991), the enzyme is capable of reacting with secondary alcohol groups (Sonnet, 1988; Vorderwülbecke et al., 1992). The degree to which various substrates impose steric constraints upon lipases in microaqueous media appears to be enzyme source specific (Wang et al., 1988; Nishio et al., 1988; Sonnet et al., 1993; Santaniello et al., 1993).

Other studies, using substrates different from those selected here, have made corroborating findings in that ac(et)yl ester cosubstrates with good leaving groups serve as efficient ac(et)yl donors for transfer reactions to alcohol acceptors as catalyzed by *C. rugosa* (Engel, 1992), *R. miehei* (Berger et al., 1992), and *Pseudomonas fragi* 22.39B (Nishio et al., 1988). Steric factors have been shown to be of paramount importance in determining the substrate selectivities of some lipases (Sonnet et al., 1993; Bevinakatti and Banerji, 1988).

The results reported in Table 1 reflect *net* extents of reaction. However, we contend that reaction revers-

Table 1. Reactivity of Selected Acetyl Donors by Acyl Transfer with 1-Undecanol and Acyl Exchange with Olive Oil Acylglycerols

acetyl donor (CH_3COR_1), where R_1 is	pK_a[c] of HR_1	acyl transfer,[a] % completion of reaction[d]	acyl exchange[b]			
			μmol of MAcDAG formed	μmol of DAcMAG formed	μmol of acetate exchanged	% completion of reaction[e]
$-O-CO-CH_3$	4.8	100	ND[g]	ND	0	0
$-O-CH=CH_2$	IRR[f]	92	15	ND	15	0.59
$-O-C_6H_5$	9.8	63	29	ND	29	1.2
$-O-CH_2-C_6H_5$	15.4	34	266	60	386	15
$CH_2-O-CO-CH_3$ $CH-O-CO-CH_3$ $-O-CH_2$	*14.4–16.1*	14	595	480	1075[h]	21[i]
$-O-(CH_2)_5CH_3$	≥16.1	8.8	230	56	342	13
$-O-CH_2CH_3$	15.9	7.5	290	98	486	19
$-O-(CH_2)_2CH(CH_3)_2$	*>16.1*	3.9	212	45	302	12
$-O-C(CH_3)=CH_2$	IRR[f]	3.2	52	11	74	2.9
$-O-C_6H_{11}$ (cyclohexyl)	*>17.1*	0	11	ND	11	0.43

[a] Acyl-transfer reaction mixtures contained about 2.5 mmol of acetyl groups for each donor substrate and 2.5 mmol of 1-undecanol with 10% (w/w) lipozyme. Results are from two to five replicates; the average coefficient of variation was 6.9%. [b] Acyl-exchange reaction mixtures contained about 2.5 mmol of acetyl groups for each acetyl donor substrate and olive oil to make up the balance of 1 g of substrate mixture, with 10% (w/w) lipozyme. Olive oil ranged from 0.62 to 0.81 g, or 2.1–2.8 mmol acyl groups, based on an average triacylglycerol molecular weight of 873 calculated from the fatty acyl composition of olive oil (Nawar, 1985). Results are from two to five replicates; the average coefficient of variation was 9.2%. [c] Known pK_a values, or those used for estimating others, were taken from Serjeant and Dempsey (1979). Estimates (in *italics*) of range or minimum pK_a values were based on those values known for glycerol (14.4), 1-propanol (16.1), 1-butanol (16.1), 2-propanol (17.1), and 2-butanol (17.6) and the tendency for increases in alkyl-chain length and branching to increase pK_a. [d] Percent completion of reaction was determined by formation of undecanyl acetate after 3 h of incubation at 60 °C relative to the initial level of acetyl groups in the donor substrates. [e] Percent completion of reaction was determined by the level of acetyl groups in MAcDAG and DAcMAG after 24 h of incubation at 60 °C relative to the initial level of acetyl groups in the donor substrates. [f] Leaving group is considered to confer an irreversible nature to the reaction (Wang et al., 1988; Santaniello et al., 1993); however, pK_a values for acetaldehyde and acetone are 13.5–13.6 and 20.0, respectively (Serjeant and Demspey, 1979). [g] ND, not detected. [h] Micromoles of MAcDAG and DAcMAG formed. [i] Since one acyl-exchange episode yields one each of DAcMAG and MAcDAG, percent reaction yield was calculated as 100 × ½(μmol of glycerol as DAcMAG and MAcDAG) ÷ 2500 μmol of acetyl groups originating from triacetin.

ibility is minimal in the reaction systems evaluated. In cases when extensive reaction took place, the corresponding alcohol leaving groups were weak nucleophiles relative to any unreactive undecanol, minimizing reversibility of the reaction. Likewise, the intended product of the reaction, undecanyl acetate, would be expected to be weakly reactive with the native enzyme in forming the acetyl enzyme intermediate (Scheme 1, steps 1 and 2), because undecanol would be a relatively poor leaving group. In cases when a limited degree of reaction took place, undecanol was in substantial molar excess of other alcohols of similar nucleophilic strength, making reaction reversibility unfavorable.

Hydrolysis in the reaction mixtures was very limited, as all of the reactive acetyl groups derived from acetic anhydride could be accounted for in undecanyl acetate, and acetic anhydride is extremely moisture-sensitive. In addition, over 90% of the acetate originating from vinyl acetate was accounted for as undecanyl acetate.

Acetyl Donor Preferences for Acetyl/Acyl-Exchange Reactions. In spite of a number of studies done on acetyl donor selectivities in acyl-transfer reactions, no similar studies could be found for reactions designed for acyl-exchange processes. This prompted an evaluation of the same acetyl donors used for acyl-transfer reactions in mixtures configured for ac(et)yl exchange with olive oil TAG (Table 1).

Because ac(et)yl-exchange reactions are reversible, the results reported in Table 1 represent net extents of reaction after 24 h of incubation. Furthermore, we measured only the appearance of acetyl groups in AG originating from olive oil, representing only half of the exchange reaction. We could not determine to what extent *true exchange* took place or to what extent a reciprocal appearance of acyl groups esterified to the alcohol functional group of the original acetyl donor occurred (*viz.*, quantitative analysis of alkyl ester coproducts was not feasible—see Experimental Procedures). In the special case of triacetin as cosubstrate with olive oil, a single ac(et)yl-exchange episode initially creates one each of the MAcDAG and DAcMAG species, whereas for all other acetyl donors a single exchange episode initially creates a single MAcDAG species. Thus, to allow for a normalized comparison of reactivity of triacetin with the other acetyl donors, the yield of (MAcDAG + DAcMAG) with triacetin as cosubstrate was halved (see Table 1 footnotes for details).

All reactions were confirmed to be enzymic with the exception of using acetic anhydride as cosubstrate. Vinyl and phenyl acetates were poor substrates for acetylating olive oil AG. These acetyl donors also have corresponding alcohol functional groups of the weakest nucleophilic character among the substrates evaluated. Ethyl acetate and triacetin were the best substrates, followed by the similarly reactive benzyl, hexyl, and isoamyl acetates, for ac(et)yl exchange with olive oil TAG. Isopropenyl and cyclohexyl acetates were poorly reactive in ac(et)yl-exchange reactions. Steric and/or rotational hindrance may play a role in the relative lack of reactivity of the latter two acetates.

The reactivity of vinyl and isopropenyl acetates strictly according to Scheme 2 is not likely because the corresponding leaving groups ($HO-R_4$), acetaldehyde and acetone, respectively, would not be expected to be reactive as acyl acceptors as shown in step 3a (Wang et al., 1988; Santaniello et al., 1993). Most likely, the limited reactivity observed is conferred by initial formation of the acetyl enzyme intermediate and acetaldehyde and acetone as leaving groups, followed by displacement of the acetyl group from the enzyme by the DAG (step 3b, Scheme 2). The extent to which this pathway can proceed is restricted by the steady-state availability of DAG species to displace acetate from the acetyl enzyme intermediate.

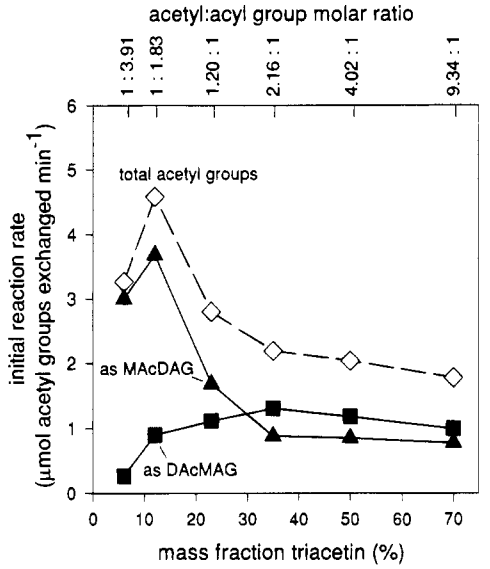


Figure 3. Effect of triacetin/olive oil mass ratios on initial rates of ac(et)yl exchange. Reaction mixtures contained different mass ratios of triacetin and olive oil in a 1.0 g substrate mixture and 0.10 g of lipozyme, with incubation at 60 °C for 30 min. Equivalent acetyl/acyl group molar ratios were calculated on the basis of molecular weights, using an average molecular weight for olive oil TAG of 873, and by assuming that only the *sn*-1,3 sites of TAG substrates are reactive. Abbreviations are the same as in Figure 1. Results are from two or three experiments, and the average coefficient of variation was 11%.

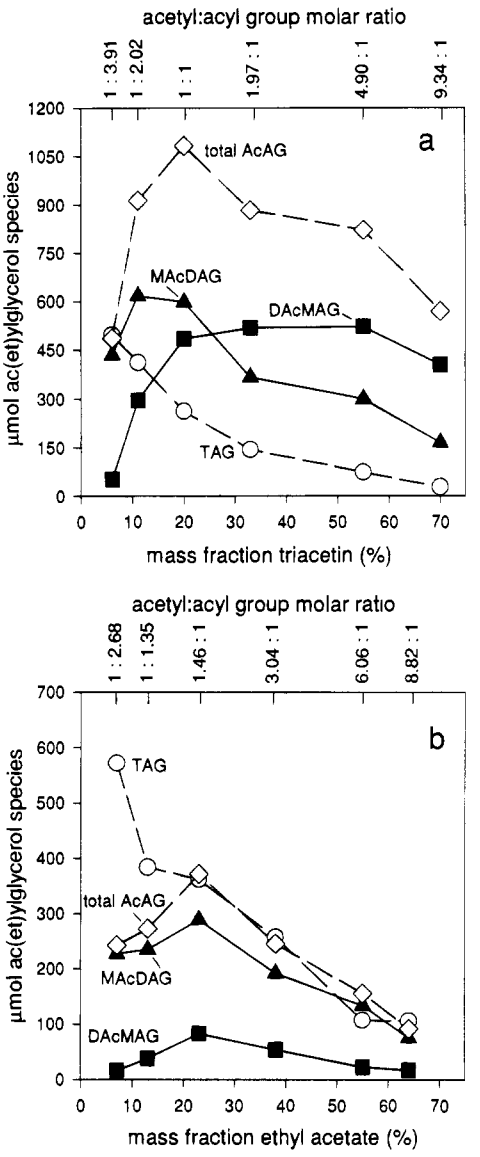


Figure 4. Effect of acetyl donor/olive oil mass ratios on extent of ac(et)yl exchange. Reaction mixtures contained different mass ratios of (a) triacetin or (b) ethyl acetate and olive oil in a 1.0 g substrate mixture and 0.10 g of lipozyme, with incubation at 60 °C for 24 h. Acetyl/acyl group molar ratios were calculated as in Figure 3. Abbreviations: same as in Figure 1; AcAG, (MAcDAG + DAcMAG). Results are from two or three experiments, and the average coefficients of variation were 8.7% and 9.6%, respectively, when triacetin and ethyl acetate were used as acetyl donor.

A pattern of relative reactivity of the selected substrates in ac(et)yl-exchange reactions with olive oil TAG seemed to emerge. Substrates that were *of similar reactivity as triacetin* with undecanol were most conducive to promoting ac(et)yl exchange with olive oil TAG. Assuming triacetin and olive oil TAG are similarly reactive with alcohol functional groups, such as the enzyme–Ser–OH group, it follows that cosubstrates yielding a balanced mixture of ac(et)yl enzyme intermediates would be most supportive of exchange reactions. In contrast, cosubstrates of disparate reactivities toward the enzyme–Ser–OH group would be expected to yield one ac(et)yl enzyme species in much greater excess than the other. The limited steady-state levels of one ac(et)yl enzyme species, and its corresponding alcohol acceptor (either DAG or HO–R_4 in Scheme 2), would restrict the progress of ac(et)yl-exchange reactions. Thus, it is concluded that substrates best suited for acyl-exchange processes are those of similar acylating reactivities with the enzyme of choice. Previous studies found that acyl-exchange reactions with TAG were most efficacious, in descending order, for other TAG > fatty acid methyl ester > fatty acid, as cosubstrate (Elliott and Parkin, 1991; Kuo and Parkin, 1993).

On the basis of these results, triacetin and ethyl acetate were evaluated further as cosubstrates in characterizing ac(et)yl-exchange reactions with olive oil, the latter of which was used as a model TAG-rich oil. The rationale for doing this was that (1) both acetyl donors were good substrates for acyl exchange (Table 1) and (2) both are compatible for use in foods.

Effect of Substrate Levels. Ac(et)yl-exchange reactions were evaluated over a range of cosubstrate mass ratios such that molar ratios of reactive acetyl/acyl groups ranged from 1:3.91 to 9.34:1 for a triacetin/olive oil mixture and from 1:2.68 to 8.82:1 for an ethyl acetate/olive oil mixture. Initial rate analysis (based on 30 min of incubation) was done only for formation of DAcMAG and MAcDAG with the triacetin/olive oil mixture (Figure 3). Greatest initial rates of ac(et)yl exchange were observed at a triacetin/olive oil mass ratio of 0.12:0.88 (acetyl/acyl group ratio of 1:1.83). This optimum may represent a compromise between the enzyme being more selective toward long-chain fatty acids (Huge-Jensen et al., 1987) and having a sufficient pool of acetyl groups to exchange with olive oil TAG and form AcAG. As expected, as the mass percent of increased, so did the ratio of DAcMAG to MAcDAG in the product mixture.

The extent of reaction and proportion of MAcDAG/DAcMAG formed after 24 h of reaction was also dependent on the initial mass ratios acetyl/acyl donors (Figure 4). For a triacetin/olive oil mixture, a mass ratio of 0.20:0.80 (molar ratio of *sn*-1,3 acetyl/acyl groups of 1:1) yielded the greatest levels of AcAG, although near-maximum levels were also formed at cosubstrate mass ratios of 0.11:0.89 to 0.55:0.45 (Figure 4a). On the basis

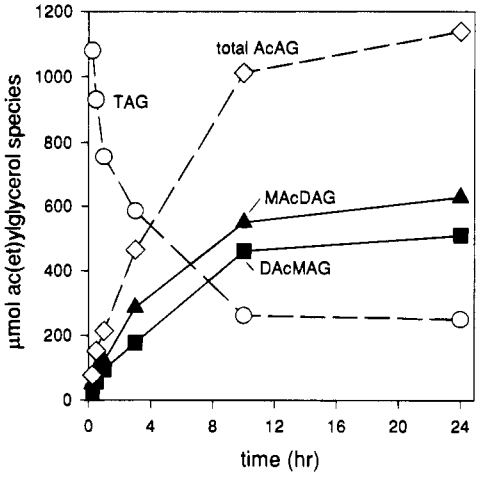


Figure 5. Progress curve for ac(et)yl exchange between triacetin and olive oil. Abbreviations are the same as in Figure 4. Reaction mixtures contained 0.2 g of triacetin, 0.8 g of olive oil, and 0.10 g of lipozyme, with incubation at 60 °C. Results are from three experiments, and the average coefficient of variation was 8.4%.

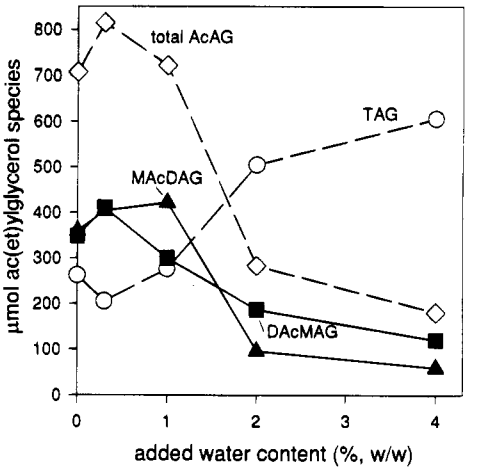


Figure 6. Effect of added water on extent of ac(et)yl exchange between triacetin and olive oil. Abbreviations are the same as in Figure 4. Reaction mixtures contained 0.50 g of olive oil, 0.50 g of triacetin, and 0.10 g of lipozyme, with incubation for 3.5 h at 60 °C. Results are from three experiments, and the average coefficient of variation was 9.6%.

of the *sn*-1,3 regiospecificity for the enzyme and the acetyl/acyl group molar ratios, theoretical maximum yields of AcAG (MAcDAG + DAcMAG) were calculated for each reaction condition. Yields ranged from 58% to 90%, with the greatest yield observed at a 0.11:0.89 mass ratio triacetin/olive oil. Varying the mass ratios of these cosubstrates allowed for product mixtures with a range of MAcDAG to DAcMAG molar ratios of 8.35–0.41 to be produced.

With ethyl acetate as acetyl donor cosubstrate with olive oil, a mass ratio of 0.23:0.77 [molar ratio of acetyl/acyl(*sn*-1,3) groups of 1.46:1] yielded the greatest levels of AcAG (Figure 4b). Substantial levels of AcAG were also produced at cosubstrate mass ratios of 0.07:0.93 to 0.38:0.62, and over this range theoretical maximum yields of AcAG (MAcDAG + DAcMAG) were 37–50%, with the greatest yield observed at a 0.23:0.77 mass ratio of ethyl acetate/olive oil. Varying the mass ratios of these cosubstrates allowed for product mixtures with a range of MAcDAG to DAcMAG molar ratios of 14.5–3.49 to be produced.

A progress curve of the reaction was developed with a cosubstrate mixture of 0.20:0.80 (w/w) of triacetin/olive oil (acetyl/acyl group molar ratio of 1:1; Figure 5). Under the conditions used, the reaction appeared to reach equilibrium within about 10 h. There also appeared to be a slight preference in forming MAcDAG over DAcMAG, even though theoretical yields of each are identical. This may be caused by a subtle TAG species specificity (Jensen et al., 1990) of the enzyme used.

Effect of Added Water. Maximum formation of AcAG species after 3.5 h of reaction was observed at 0.3% (w/w) added water, with near-maximum yields attained at 0–1% added water (Figure 6). This corresponds to 1–2% total water in the system or about 0.5–1.0 M water based on the total volume of the reaction mixture, including the water content (about 10%) of the enzyme. At 2–4% added water, reaction yields declined, and this was associated with a reduced ability to transform olive oil TAG. Our results compare favorably with those of Leitgeb and Knez (1990), where, by our calculations, the total water content optimal for lipozyme-mediated butyl oleate synthesis at 50–70 °C is about 1.1–1.8 M.

The effects of the greater levels of water evaluated were probably manifest as a shift in reaction equilibrium away from ac(et)yl exchange and toward hydrolysis. The extent of hydrolysis, based on molar proportion of DAG (relative to initial TAG levels), increased from about 9% at no added water to 21% at 1% added water. However, at 2% added water the extent of hydrolysis was limited to about 15%, indicating a general attenuation in enzyme activity. Enzyme activity could have been impaired by the caking or flocculation of the catalyst observed at 2–4% added water.

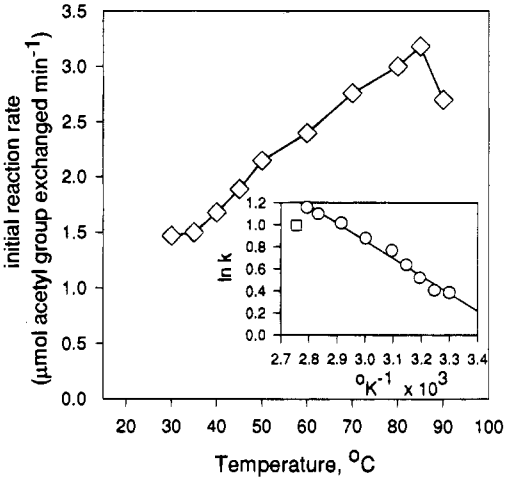


Figure 7. Effect of temperature on the initial rate of ac(et)yl exchange between triacetin and olive oil. Conditions are as described in Figure 5, except that temperature was varied and incubation time was 30 min. Results are from three experiments, and the average coefficient of variation was 3.1%. The inset reflects transformation of data to an Arrhenius plot, where the data point representing 90 °C (open square) were ignored for the purpose of linear regression analysis.

Effect of Temperature. The effect of temperature on the progress of ac(et)yl-exchange reactions was evaluated using triacetin and olive oil as cosubstrates (Figure 7). Maximum initial rates of DAcMAG formation were observed at 80–85 °C; this was similar to the thermal response of the same enzyme when used to mediate butyl oleate synthesis (Habulin and Knez, 1993). In contrast, the thermal stability of a crude *R. miehei* preparation in aqueous medium is limited to about 45 °C (Moskowitz et al., 1977). The thermal stability of lipozyme under the reaction conditions used was also evident with extended incubation times. After

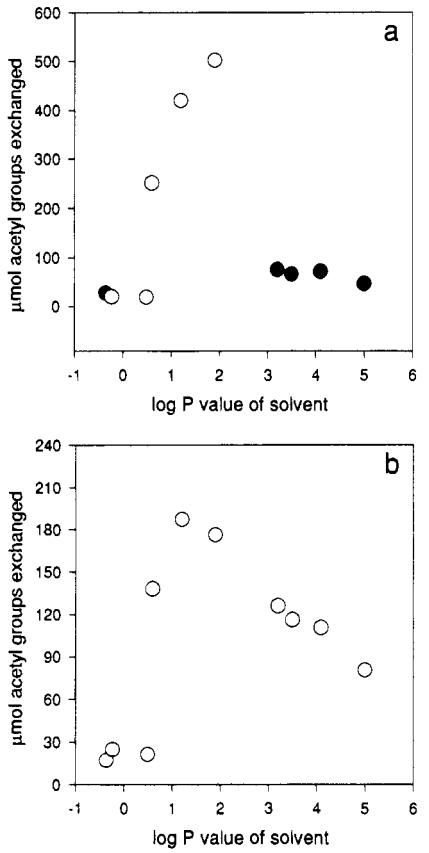


Figure 8. Effect of solvent suitability for supporting ac(et)yl exchange between triacetin or ethyl acetate and olive oil. (a) Reaction mixtures contained 0.33 g of triacetin, 0.67 g of olive oil and 0.10 g of lipozyme in 5 mL of solvent. (b) Reaction mixtures contained 0.23 g of ethyl acetate, 0.77 g of olive oil, and 0.12 g of lipozyme in 5 mL of solvent. Mixtures were incubated at 60 °C for 24 h. The extent of reaction was based on levels of diacetylmonoacylglycerol formed; 1 mol formed represents a single acetyl-group exchange for the triacetin/olive oil mixture and two acetyl-group exchanges for the ethyl acetate/olive oil mixture. Solvents used [and corresponding log *P* values, taken from Laane et al. (1987)] were acetonitrile (−0.36), acetone (−0.23), tetrahydrofuran (0.49), *tert*-butyl alcohol (0.80), *t*-butyl methyl ether (1.2), isopropyl ether (1.9), cyclohexane (3.2), hexane (3.5), heptane (4.1), and isooctane (5.0). Open and solid symbols indicate, respectively, that single and dual liquid phases were present. Results are from three experiments, and the average coefficients of variation were 8.6% and 8.4% in (a) and (b), respectively.

10 and 24 h, the respective yields of DAcMAG were 540 and 528 μmol at 80 °C, compared to 490 and 570 μmol at 60 °C and 318 and 530 μmol at 40 °C. Transformation of the initial rate data to an Arrhenius plot for the temperature range 30−80 °C gave a linear response corresponding to an activation energy of 3.84 kcal/mol. This is similar to the value of 2.28 kcal/mol reported by Habulin and Knez (1993) for the same enzyme in microaqueous media. Enhanced thermal stability in microaqueous media is a general response of enzymes (Zaks and Klibanov, 1984; Klibanov, 1986; Dordick, 1989).

Effect of Solvent. The use of organic solvents as a continuous phase was evaluated for the ability to support ac(et)yl-transfer reactions with TAG (Figure 8a). The extent of reactions after 3.5 h was dependent on the log *P* (*P* is the partitioning coefficient of the solvent between 1-octanol and water) value of the solvent and the nature of the acetyl donor. Using triacetin as acetyl donor, a sharp optimum for reactivity was observed for solvents of log *P* values 1−2, where a single liquid phase appeared to exist (Figure 8a). Phase behavior alone was not accountable for these observations, as tetrahydrofuran and acetone promoted a single liquid phase but were not very supportive of reaction. Using ethyl acetate as the acetyl donor, a similar optimum was observed with solvents of log *P* values 1−2, and all solvents evaluated promoted a single liquid phase (Figure 8b). However, the optimum was not as sharp as it was for triacetin, and substantial activity was observed with ethyl acetate when solvents of log *P* values up to 5 were used. This difference in range of log *P* values for solvents supporting reactivity may be partially based on substrate polarity. Triacetin (log *P* of −0.075; Yang et al., 1994b) is more polar than ethyl acetate [calculated log *P* of 0.77, as per Rekker and Mannhold (1992)], and a positive correlation between capability of nonpolar solvents to support biocatalysis with increasing nonpolar nature of substrates has been observed (Yang et al., 1994b).

The activity profile as a function of solvent log *P* value was also dependent on the enzyme source. *tert*-Butyl methyl and isopropyl ethers were only 47−76% as supportive of activity of the *Pseudomonas* sp. (type PS-30) lipase as they were for lipozyme, even though both enzymes exhibited similar dependencies of activity on solvent log *P* values (data not shown). This enzyme source specific behavior may be attributable simply to differences in purity of the enzyme preparations. Phase behavior of reaction systems appeared to have only secondary influence on reactivity, although this effect may also be enzyme source specific (Yang et al., 1994a).

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