FA Selectivity of Lipases in Acyl-Transfer Reactions with Acetate Esters of Polyols in Organic Media

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ABSTRACT: FA reaction selectivity of *Burkholderia cepacia, Rhizomucor miehei,* and *Candida antarctica* fraction B lipases was compared between acyl-transfer and esterification reactions. Multicompetitive reaction mixtures containing a series of *n*-chain FA (a C_4–C_{18} series; and a $C_{18:X}$ series, where X = 0–3 double bonds) and a single acetate ester co-substrate [triacetin, 1,2-propanediol (1,2-PD) diacetate, and 1,3-PD diacetate] were studied in *tert*-butyl methyl ether at an a_w of 0.69. For *B. cepacia* lipase, FA optima for C_8, C_{16}, and $C_{18:2}$ were observed in all reactions with 1.0- to 5.9-fold differences in FA selectivity. For *R. miehei* lipase, an optimum for C_8 FA was observed in all reactions with 1.2- to 6.7-fold differences in FA selectivity. For *C. antarctica* lipase, FA optima for C_8/C_{10} were observed in all reactions with 1.0- to 2.8-fold differences in FA selectivity. FA selectivities were broadly modulated upon changing from free polyol to acetate ester co-substrates for *B. cepacia* and *R. miehei* lipases, whereas FA selectivity modulations were more specific upon this change in reaction configuration for *C. antarctica* B lipase. For all lipases, reactivity toward unsaturated $C_{18:X}$ FA was enhanced in acyl-transfer relative to esterification reactions with these polyol co-substrates.

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Widespread applications of lipase-catalyzed reactions in organic media for the food, pharmaceutical, and olechemical industries are believed to exist (1–4). The fundamental advantage of reaction selectivity favors the use of lipases over chemical processes for transforming acylglycerols into value-added specialty products, commonly referred to as structured lipids (5,6). To fully exploit the capabilities of lipases for preparing structured lipids, an understanding of the factors that confer or control reaction selectivity with regard to the strategic and differential positioning of acyl groups along the glycerol backbone is necessary. Although there have been several studies on substrate selectivity of lipase-catalyzed reactions in organic media (7–10), there has been only a limited systematic evaluation of the selectivity characteristics of lipases and how they may be controlled in reactions relevant to acylglycerol modification.

Our previous studies have focused on "intrinsic" chain length selectivity toward FA substrates in esterification reactions by various lipolytic enzymes with glycerol and diol analogs (11–13). In these studies, the alcohol functional groups of the glycerol backbone were observed to differentially modulate FA selectivities for all lipases examined. To further understand how co-substrate structural features may affect lipase reaction selectivity, the present study evaluated how FA selectivity changed for reactions with free alcohols compared to their acetate esters for glycerol and diol analogs. These alcohol co-substrates were selected to model the reactivities and functional units (both free alcohol and ester groups) of the *sn*-glycerol backbone in reactions relevant to acylglycerol modification.

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EXPERIMENTAL PROCEDURES

Materials. The lipases evaluated were from *Burkholderia cepacia* [formerly *Pseudomonas* (14)], *Rhizomucor miehei* ("Chirazyme L-9, c.-f., dry," immobilized on a macroporous anion-exchange resin; equivalent to "Lipozyme IM"), and *Candida antarctica* fraction B ("Chirazyme L-2, c.-f., C2, lyo.," immobilized on a macroporous acrylic resin; equivalent to "Novozyme 435"). Chirazyme preparations were gifts from Roche Diagnostics/Boehringer Mannheim Co. (Indianapolis, IN) and the *B. cepacia* lipase preparation ("PS30," powder) was purchased from Amano Enzyme USA Co. (Lombard, IL). All enzyme preparations were used as received without further treatment. *cis*-9-Octadecenoic and *cis*-9,12,15-octadecatrienoic acids were obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). Disodium hydrogen phosphate dihydrate and dodecahydrate, and 1,2-propanediol diacetate were obtained from Fluka Chemie AG (Buchs, Switzerland). TLC plates (Whatman PE Sil G/UV, 250-μm thickness) were obtained from Fisher Scientific (Chicago, IL). All other chemicals and HPLC- or GC-grade solvents used were obtained from Aldrich Inc. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO).

Synthesis of 1,3-propanediol (1,3-PD) diacetate. 1,3-PD diacetate was prepared by the acetylation of 1,3-PD with acetic anhydride and isolated by vacuum distillation (15,16). A modified procedure was used where 140 mL acetic anhydride was slowly added into a gently stirred mixture of 30 g 1,3-PD and 3 mL concentrated H_2SO_4. The mixture was gently refluxed for 1 h. The resulting solution was cooled to 21–24°C and poured into 100 mL cold water (4°C) followed by extraction into three 100-mL portions of ether. The ether extracts were pooled and neutralized with 5% aqueous sodium bicarbonate until the aqueous layer tested as alkaline on litmus paper. The ether extract was dried over 5 g anhydrous magnesium sulfate, and solvent was removed by rotary evaporation with the 1,3-PD diacetate isolated by vacuum distillation (65–70ºC at 30 mm Hg, 60–70% yield). Identity and purity were confirmed by ^{1}H NMR (model AM-300

NMR spectrometer; Bruker Instruments, Inc., Billerica, MA) (300 MHz, $CDCl_3$): δ 1.97 (*p,* 2H, *J* = 6.3 Hz), 2.06 (*s,* 6H), 4.15 (*t,* 4H, *J* = 6.3 Hz), based on previously reported spectra (17), and silica gel TLC (R_f = 0.56 for the diester, 0.20 for the monoester) using petroleum ether/ether/acetic acid, 65:35:1 by vol, as the developing solvent system.

Reaction parameters. Reaction conditions were similar to those described by Lee and Parkin (12), unless stated otherwise. Reaction mixture design was based on multicompetitive substrate systems (18). Each reaction mixture contained multiple FA substrates at 50 mM and a single alcohol co-substrate at 500 mM in a total volume of 20 mL *tert*-butyl methyl ether (*t*BME) as the continuous phase. Two groups of FA substrates were evaluated separately: One included the homologous series of even carbon number *n*-chain saturated FA (C_4–C_{18}, total 400 mM FA); the other contained a series of *n*-chain $C_{18:X}$ FA (where X = 0–3 double bonds, total 250 mM FA). A reference saturated FA (C_8) was also included in the $C_{18:X}$ FA group to allow for comparisons of selectivities between groups of FA substrates. Although the relative specificity constants (α-values) determined for any FA common to both series of FA substrates should be identical (18), on some occasions they were not in our studies. This anomaly may be caused by physical differences in the two FA substrate systems used, since one was a saturated series and the other was largely the unsaturated series, but this prospect was not evaluated further in the present study.

In comparing FA selectivity between acyl-transfer and esterification reactions, the alcohol co-substrates used were triacetin, 1,2-PD diacetate, and 1,3-PD diacetate in the former reaction configuration, and the corresponding free polyols in the latter reaction configuration. Acetate esters were selected as the simplest form of alkanoic ester that could exist in natural lipids and to allow focus on the effect of an ester bond along the polyol backbone on FA selectivity without any confounding influence of acyl chain length. A previously published study (12) was used as the source of historical selectivity data (for C_4–$C_{18:0}$ FA) for esterification reactions to allow for a complete comparison of FA selectivity between reaction types. Periodic repetition of some of these earlier studies indicated that these historical results were reproducible (validated) for the comparisons made in this paper.

Water activity (a_w) was controlled at 0.69 by adding 1.0 g each of the di- and heptahydrates of $Na_2HPO_4 \cdot (H_2O)_x$ (19). Reaction mixtures were preincubated at 35°C for 30 min in an orbital shaker at 300 rpm, and enzyme (0.05 to 0.7 g; preconditioned with 0.4 g each salt hydrates and 10 mL *t*BME) was added to initiate the reaction.

Analysis of reaction progress. Reaction progress was monitored by removing subsamples at appropriate time intervals and derivatizing by transmethylation (20) as described earlier (12), unless stated otherwise. The FAME were analyzed using a Hewlett-Packard (HP) 6890 series gas–liquid chromatograph (Hewlett-Packard, Wilmington, DE) equipped with a FID, and HP-5 (cross-linked 5% phenyl methyl siloxane; 30 m × 0.32 mm i.d.; 0.25-μm film thickness; Hewlett-Packard) and HP-INNOWAX (cross-linked polyethylene glycol; 30 m × 0.32 mm i.d.; 0.5-μm film thickness; Hewlett-Packard) capillary columns for saturated and $C_{18:X}$ FAME, respectively. The temperature program for the analysis of saturated C_4–C_{18} FAME was the same as described by Lee and Parkin (12). The temperature program for the analysis of $C_{18:X}$ FAME was: initial hold at 50°C for 2 min, ramp from 50 to 220°C at 18°C min^{-1}, then from 220 to 250°C at 10°C min^{-1}, and then hold at 250°C for 4 min.

Substrate selectivity analysis. FA selectivity was evaluated by a competitive factor (α value), which is proportional to the specificity constant, V_{max}/K_M, based on the theory advanced by Deleuze *et al.* (18) and Rangheard *et al.* (21), and as used by Berger and Schneider (22) and in our previous study (12). In the present study, C_8 was taken as the reference substrate and assigned an α-value of 1. The greater the α value, the greater the selectivity (V_{max}/K_M) for a particular FA substrate. For each enzyme, a range (minimum to maximum) of difference in FA reactivity relative to the rate of the reaction with the reference FA (C_8) observed under all reactions conditions was calculated and expressed as "fold-differences in selectivity." This range was calculated by taking the ratio of α-values between the reference FA (C_8) and each of the two other FA species in the mixture with α-values most similar (to obtain the minimum value) and dissimilar (to obtain the maximum value) to that of FA C_8, where a ratio of 1.0 indicates no difference in selectivity or α-value.

RESULTS AND DISCUSSION

Comparative lipase reactivities with different polyalcohol acetates. Initial rates of lipase-catalyzed acyl-transfer reactions were dependent on the specific alcohol acetate used as co-substrate (Table1). These rates for acyl-transfer reactions with alcohol acetate co-substrates were 0.55 to 48% of those observed for esterification reactions for the same FA mixtures and free glycerol or diol co-substrates (12). In addition, the *B. cepacia* lipase reacted fastest with 1,2-PD diacetate, whereas the *R. miehei* and *C. antarctica* lipases reacted fastest with 1,3-PD diacetate for acyl-transfer reactions (Table 1). In contrast, in esterification reaction mixtures the alcohol co-substrate preferences for the *B. cepacia* and *R. miehei* lipases were reversed (12). The primary difference between esterification and acyl-transfer reactions is that the alcohol acetate ester must first be hydrolyzed in the latter system to yield a free alcohol

TABLE 1
Initial Reaction Rates of Acyl-Transfer Reactions of Lipases with Various Acetate Ester Co-substrates

	Initial reaction rate (nmole min^{-1} mg^{-1})[a]		
Lipase	Triacetin	1,2-PD diacetate	1,3-PD diacetate
Burkholderia cepacia	3.46 (0.18)[b]	4.82 (0.48)	0.93 (0.0055)
Rhizomucor miehei	3.59 (0.065)	6.25 (0.034)	12.8 (0.091)
Candida antarctica B	3.75 (0.026)	37.1 (0.20)	109 (0.44)

[a]Results are expressed as means for rates of C_8 acyl-transfer reactions at a_w 0.69, determined over the first 10 min of reaction where linearity was confirmed, from two or three experiments with a CV of about 10%. 1,2-PD diacetate: 1,2-propanediol diacetate; 1,3-PD diacetate: 1,3-propanediol diacetate.

[b]Values in parentheses represent the ratio of initial reaction rates for acyl-transfer/esterification reactions (using previously reported data for esterification reactions with *B. cepacia* and *R. miehei* lipases, from Table 1 in Ref. 12).

pool before net acyl-transfer can take place *via* a subsequent esterification reaction between FA and alcohol (23–26).

Large differences between acyl-transfer and esterification reaction rates (small numbers in parentheses in Table 1) may be accounted for in many ways. If reaction between the enzyme and the alcohol acetate is faster than that with the FA pool, then the steady-state acyl-enzyme pool may be almost exclusively in the acetyl-enzyme form, providing little opportunity for reaction with FA from the original FA pool. This may be the case for *B. cepacia* lipase using the 1,3-PD diacetate, since *B. cepacia* lipase is very reactive with acetate esters (27) and readily accepts 1,3-PD as an alcohol co-substrate (12). We have previously suggested and provided evidence for the need to maintain a balance between acetyl- and acyl-enzyme pools to facilitate acyl-transfer reactions (25,26,28). Another reason for any large differences in reaction rates between esterification and acyl-transfer reactions is that the lipase does not readily react with the alcohol acetate co-substrate relative to the free alcohol. This may explain the across-the-board differences in reactivity in the acyl-transfer and esterification reaction systems for *R. miehei* lipase, an enzyme known to be very slow in reacting with acetate/acetic acid groups (21). Lastly, since the polyalcohol acetates used in the present study would become reactive co-substrates after liberation of only one acetyl group, differences in reactivity in acyl-transfer reaction systems compared to esterification reaction systems also may be founded on differences in reactivity of monohydroxy compared to di-/trihydroxy forms of the 1,2-PD, 1,3-PD, or glycerol co-substrates. This latter feature may also be compounded by the characteristic regioselectivity of the particular lipase (10,29).

Comparative lipase selectivities with different polyalcohol acetates. (i) Burkholderia cepacia *lipase*. FA optima for C_8, C_{16}, and $C_{18:2}$ were exhibited by *B. cepacia* lipase powder in acyl-transfer reactions with all three polyol acetate ester co-substrates (Fig. 1). The fold-differences in selectivity, based on α-value comparisons between the reference FA (C_8) and the other FA in the reaction mixture, ranged from 1.0 to 5.9 among the saturated C_4–C_{18} series and up to 3.0 for the $C_8/C_{18:X}$ series. Reactions with 1,2-PD diacetate were more discriminatory against $C_{18:X}$ (X = 1–3) than were reactions with 1,3-PD diacetate and triacetin.

Relative to esterification reactions with glycerol, acyl-transfer reactions with triacetin were of enhanced selectivity toward C_{10}–C_{16} and $C_{18:X}$. A similar enhancement in selectivity toward the unsaturated members of the $C_{18:X}$ FA series was observed for esterification/acyl-transfer reactions based on 1,3-PD/diacetate. These results are consistent with the removal of a constraint (likely mediated by polar or electronic forces) in reactivity with long-chain FA imposed by the presence of the two primary hydroxyl groups (*sn*-1,3) along the propane backbone as they become esterified. Further support of this premise comes from the limited impact on long-chain FA selectivity for comparative reactions based on 1,2-PD and its diacetate. Lastly, selectivity toward saturated FA ≤ C_6 was enhanced when the alcohol substrates were changed from 1,2- or 1,3-PD to the diacetate derivatives but was diminished with the change from glycerol to triacetin as the alcohol co-substrate.

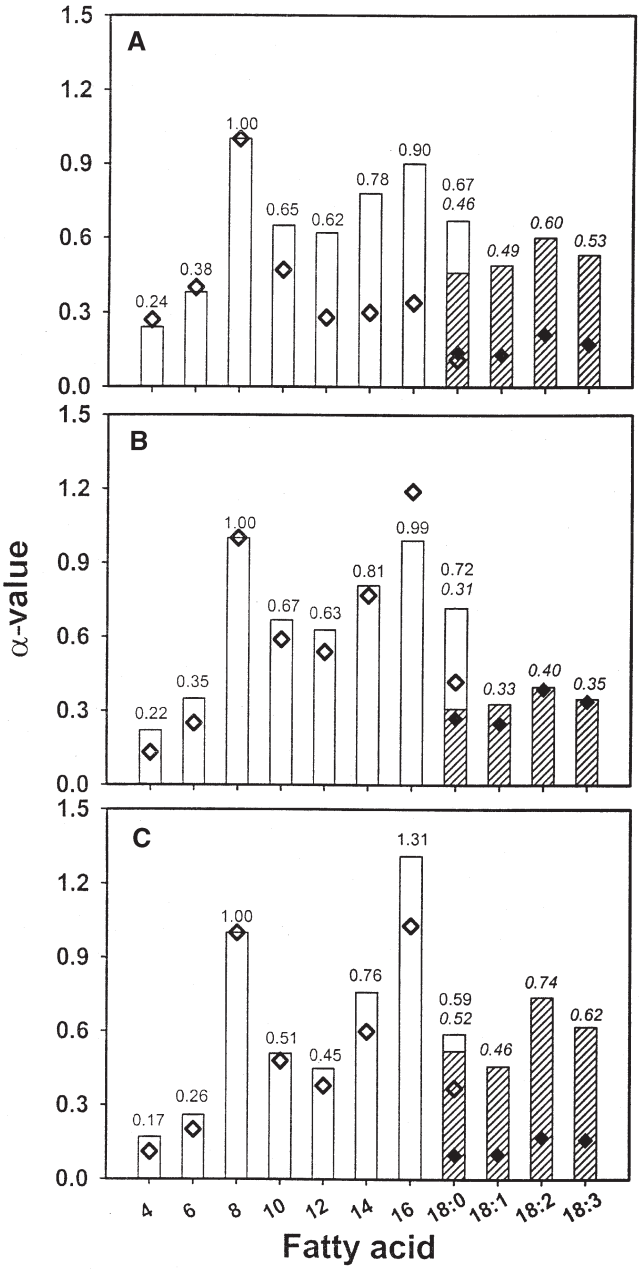

FIG. 1. α-Values for FA in acyl-transfer reactions catalyzed by *Burkholderia cepacia* lipase with (A) triacetin, (B) 1,2-propanediol (1,2-PD) diacetate, or (C) 1,3-propanediol (1,3-PD) diacetate. The open bars and hatched bars represent α-values obtained for the saturated series of FA (C_4–C_{18}) and a series of $C_8/C_{18:X}$ FA (where X = 0–3 double bonds) substrates, respectively. The α-values enumerated in *italics* are derived from experiments with the latter FA substrate series. The corresponding α-values for esterification reactions are shown as "◇" for the C_4–$C_{18:0}$ FA series (data from Ref. 12) and "◆" for the $C_{18:X}$ FA series using the corresponding free polyols as co-substrate. Each FA was used at 50 mM, with a single alcohol acceptor at 500 mM and lipase (50 to 120 mg for esterification and 500 to 700 mg for acyl-transfer) in 20 mL *tert*-butyl methyl ether at 35°C. Results are expressed as means from two or three experiments with a CV of about 6%.

(ii) Rhizomucor miehei *lipase*. FA optima toward C_8 and C_{12}–C_{18} as a group were exhibited by immobilized *R. miehei* lipase in acyl-transfer reactions with all three polyol acetate ester co-substrates (Fig. 2). All reactions were discriminatory

toward FA ≤ C_6, and little difference in selectivity toward members of the $C_8/C_{18:X}$ series of FA was consistently observed with each of (or between) the alcohol co-substrate systems, although $C_{18:3}$ was the least favorable substrate in each case. The fold-differences in selectivity, based on α-value comparisons between the reference FA (C_8) and the other FA in the reaction mixture ranged from 1.2 to 6.7 among the saturated C_4–C_{18} series, but only up to 1.7 for the $C_8/C_{18:X}$ series. Although the ordinal patterns of FA selectivity for this enzyme were not affected by the change from esterification to acyl-transfer reactions in going from polyol to polyol acetates, the degree to which the lipase discriminated among the $C_8/C_{18:X}$ series of FA substrates (the range of α values) was diminished when reactions were conducted as acyl-transfer processes.

(iii) Candida antarctica *B lipase*. FA optima for C_8/C_{10} were exhibited by immobilized *C. antarctica* B lipase in acyl-transfer reactions with all three polyol acetate ester co-substrates (Fig. 3). In acyl-transfer reactions with triacetin and 1,3-PD diacetate, $C_{18:0}$ was also preferred among FA of C_{14}–$C_{18:X}$, whereas FA selectivity among this group of FA was less evident for reactions with 1,2-PD diacetate. Relative to the reactions with the PD diacetates, acyl-transfer processes with triacetin favored reaction toward C_4/C_6 FA. The fold-differences in selectivity, based on α-value comparisons between the reference FA (C_8) and the other FA in the reaction mixture ranged from 1.0 to 2.8 among the saturated C_4–C_{18} series, and 1.5 to 2.2 among the unsaturated $C_{18:X}$ series, for acyl-transfer reactions with these polyol acetates.

Overall, reactions with all three polyol acetates were of enhanced selectivity toward the $C_8/C_{18:X}$ FA series relative to esterification reactions with the free polyhydroxy derivatives of these same co-substrates. Thus, a constraint to reactivity with $C_{18:X}$ FA in esterification reactions was apparently removed when hydroxyl groups were bound by ester linkages, similar to what was observed for the *R. miehei* lipase (Fig. 2). Otherwise, there were unique and limited modulations of FA selectivity for each of the three alcohol/acetate ester co-substrates. As the reaction configuration was changed from esterification to acyl-transfer, short-chain FA reactivity was enhanced for reactions with glycerol/triacetin, C_{12}–C_{16} reactivity was enhanced for reactions with 1,2-PD/diacetate, and C_{14}/C_{16} reactivity was diminished for reactions with 1,3-PD/diacetate (Fig. 3).

Ramifications for preparing structured glycerides. The use of lipases to prepare structured glycerides can be viewed as a multistep process, especially if one anticipates *de novo* assembly from glycerol and FA substrates. We have been working under the premise that reaction selectivity may be modulated by structural features of the co-substrates, such that FA reactivity for each successive step in assembling TAG may be differentially and partially controlled by the prevailing alcohol co-substrate (i.e., glycerol or partial glyceride) at any time during the overall synthesis. We have previously reported on how structural features of the glycerol backbone modulate lipase selectivity (11,12).

The polyol acetates used as co-substrates for the present studies are considered as representative of acylglycerol analogs present near the beginning or end (or throughout) a

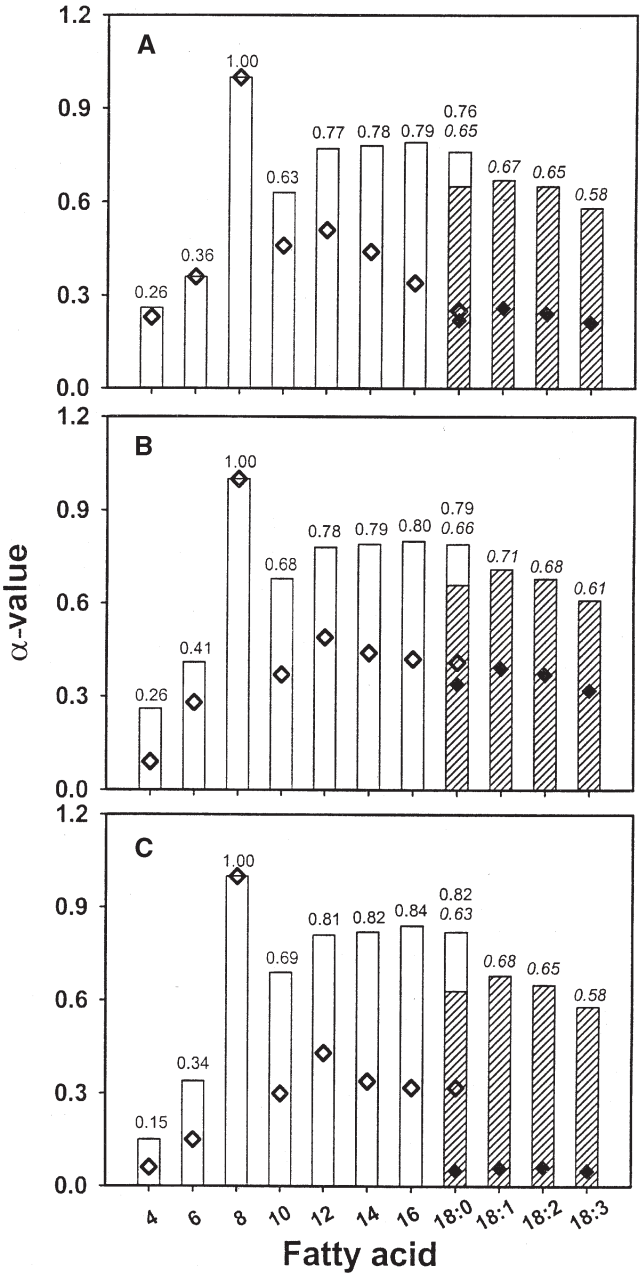

FIG. 2. α-Values for FA in acyl-transfer reactions catalyzed by *Rhizomucor miehei* lipase with (A) triacetin, (B) 1,2-PD diacetate, or (C) 1,3-PD diacetate. The legend is the same as for Figure 1, except that lipase was used at levels of 50 to 100 mg for esterification and 200 to 400 mg for acyl-transfer. Results are expressed as means from two or three experiments with a CV of about 6%. For abbreviations see Figure 1.

structured glyceride synthesis, depending on the original choice of co-substrates. In either case, the putative alcohol substrate would likely contain only a single free hydroxyl group, given that the alcohol would be the limiting substrate and steady-state levels would remain low. Thus, any comparisons of FA selectivity between esterification and acyl-transfer reactions drawn in the present study must recognize that the putative nucleophile in each case is the free polyol and the monohydroxy acetylated polyol, respectively, as well as the lower steady-state levels of alcohol nucleophile and the accumulation of acetate in the latter reaction configuration.

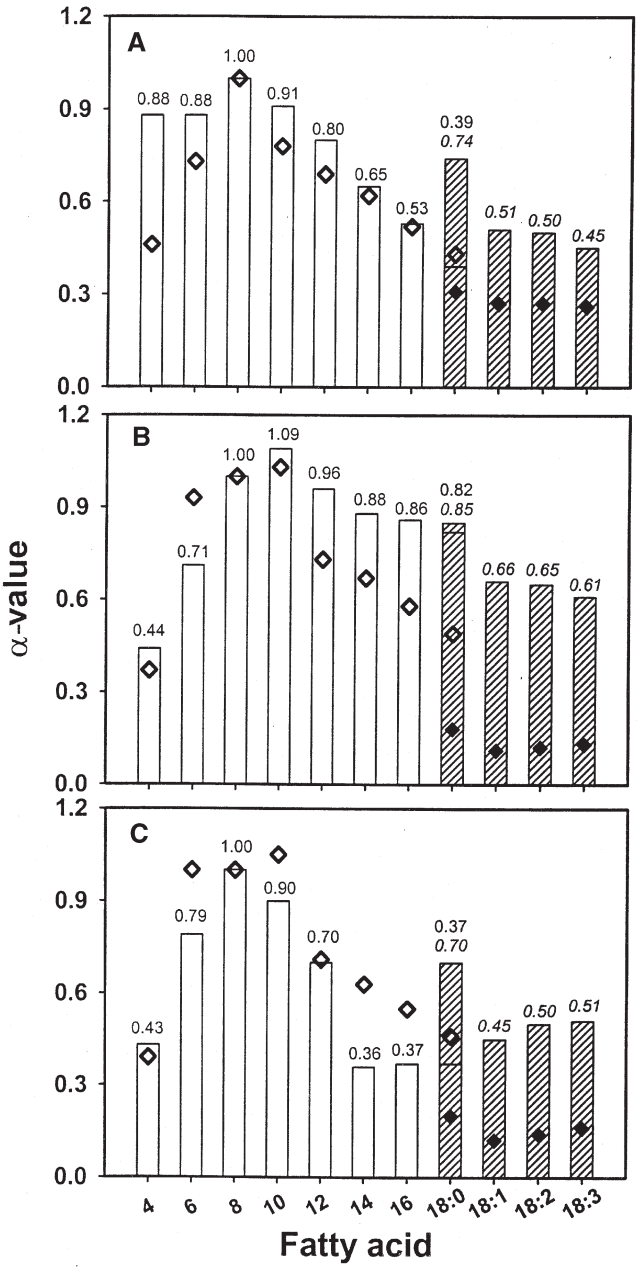

FIG. 3. α-Values for FA in acyl-transfer reactions catalyzed by *Candida antarctica* lipase B with (A) triacetin, (B) 1,2-PD diacetate, or (C) 1,3-PD diacetate. The legend is the same as for Figure 1, except that lipase was used at levels of 25 to 30 mg for esterification and 50 to 300 mg for acyl-transfer. Results are expressed as means from two or three experiments with a CV of about 5%. For abbreviations see Figure 1.

Each of the lipases exhibited a unique and characteristic pattern of FA selectivity. Each lipase was also unique in how these FA selectivity patterns were modulated by changing the reaction configuration from esterification to acyl-transfer (12; this study). Whereas ordinal patterns of FA selectivity were generally retained for all lipases, the degree of FA selectivity or ability to discriminate between FA for each enzyme changed upon modifying the reaction configuration. For *B. cepacia* lipase, there was a general loss in ability to discriminate between FA substrates, as indicated by a narrower range in α values, for the triacetin and 1,3-PD polyol diacetete, but not 1,2-PD diacetate co-substrate systems, relative to the corresponding esterification reactions. For *R. miehei* lipase, there was a loss in ability to discriminate between FA substrates, as indicated by a narrower range in α values, for all polyol acetate co-substrate systems, relative to the corresponding esterification reactions. For *C. antarctica* lipase B, there were specific rather than broad modulations in FA selectivity that were dependent on the specific polyol acetate co-substrate system used, relative to the corresponding esterification reactions.

Thus, FA selectivity of *B. cepacia* lipase was modulated by changes in the nature (free vs. esterified) of the *sn*-1,3 alcohol groups, whereas FA selectivity of the *R. miehei* and *C. antarctica* B lipases was affected by changes in the nature of the alcohol sites along the propane backbone in general. One distinction between the latter two enzymes was that changes in FA reactivity for reactions with polyol acetate esters relative to polyols were all enhanced for the *R. miehei* lipase, whereas for the *C. antarctica* B lipase, reaction selectivity was either enhanced or diminished for some FA substrates, depending on the specific alcohol co-substrate. However, in general, acyl-transfer reactions with polyol acetate co-substrates provided for enhanced reactivity over the corresponding esterification reactions with free polyols toward long-chain FA for all three lipases studied.

The results, in context of the choice of polyol/acetate co-substrates used, imply that the observed modulations in FA selectivity are based on differences in selectivity for the deacylation steps between free polyol and partially acetylated derivatives, as is observed for other alcohols (30). These differences may be founded on modulations in a polar or electronic interaction(s) between enzyme and co-substrate(s) that affect reaction kinetics. It was previously observed that FA selectivity of *R. miehei* and *C. antarctica* B lipases was modulated as (iso)propanol acetates were substituted for the corresponding free alcohol for the same FA co-substrate system (25,26). Alternatively, the accumulation of acetic acid in the acyl-transfer reaction systems also may play a role in modulating enantio- and FA selectivity as has been shown for reactions that accumulate acetate (31) and butanoate (32). Acetic acid from the hydrolysis of dodecyl acetate, catalyzed by *R. miehei* lipase in water-saturated 3-pentanone, changes the pH of the enzyme microenvironment (33), which also could influence FA selectivity. Further insight into what mechanisms are involved in modulating FA selectivity based on the results from the present study would require more focused and systematic studies of substrate derivatives, employing an approach similar to that used to study the influence of the nature of the *sn*-2-glycerol site on hydrolytic selectivity of microbial lipases (34).

A general observation made for all lipases evaluated in the present study was that reactivity toward $C_{18:X}$ FA was enhanced (in 34 of 36 instances, the other two showing no change) in reactions with any of the polyol acetates compared to reactions with the corresponding free polyols. With reference to the prospects of preparing structured glycerides, it would appear to be most favorable to attempt the incorporation of unsaturated FA into glycerides that are almost fully acylated, perhaps as a terminal step. Apparently, a constraint

of reactivity with $C_{18:X}$ FA in free polyols is diminished as the hydroxyl groups are bound in ester linkages.

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