Lipase-Catalyzed Enantioselective Acidolysis of Chiral 2-Methylalkanoates

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Lipase from *Candida cylindracea* catalyzes the acidolysis between racemic 2-methylalkanoates and fatty acids in heptane with a preference for the (S)-configurated esters. Velocity and enantioselectivity of the acyl transfer are strongly influenced by the structures of the initial substrates. The potential of this strategy for kinetic resolution of the enantiomers of chiral 2-methylalkanoates is demonstrated. Limitations of the procedure due to the reversible character of the reaction are discussed.

Acidolysis, chirality, enantioselectivity, esterification, kinetic resolution, lipase, 2-methylalkanoates, transesterification.

Lipase-catalyzed interesterification is one of the strategies to prepare lipids with desired properties that may be unobtainable by chemical procedures (1). Directed acyl exchange based on a lipase-mediated reaction between a triacylglycerol and a fatty acid (acidolysis) has been developed from a basic reaction (2,3) to a commercially applicable process (4,5). Valuable cocoa butter equivalents from low-cost starting materials are examples of target structures (6).

Lipases accept not only triacylglycerols, but also other (unnatural) esters as substrates for acidolyses (7,8). However, the exciting principle of enantioselection, widely exploited for lipase-catalyzed procedures, such as hydrolyses, esterifications and transesterifications *via* alcoholysis (9–11), had not been investigated for this type of reaction.

The first example of the enantioselective course of acidolysis was the lipase-catalyzed reaction between a chiral 2-hydroxyalkanoate and a fatty acid in organic solvent (12). The structurally related 2-methyl substituted acids and esters, respectively, are important naturally occurring flavor compounds (13), as well as valuable synthons for asymmetric syntheses of natural products with branched chain structures, such as pheromones (14). Kinetic resolution of methyl-branched substrates can be achieved by means of lipase-catalyzed esterification and hydrolysis (15,16). Preliminary investigations revealed that a biocatalyzed acidolysis of 2-methylalkanoates might be a useful alternative (17). This paper presents detailed data on the kinetic and thermodynamic parameters determining the applicability of this procedure.

EXPERIMENTAL PROCEDURES

Materials. Lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) from *Candida cylindracea*, now known as *Candida rugosa*, was purchased from Sigma (Germany). The crude enzyme preparation (L 1754) had a hydrolytic activity of 665 units per mg solid (olive oil, pH 7.2, 30 min incubation). All chemicals were obtained commercially and were of analytical grade. Heptane ($H_2O \leqslant 0.05\%$) was used as received. Solvents for liquid-solid chromatography were distilled before use.

Reaction mixtures. Racemic 2-methylalkanoate (0.5 mMol), 0.5 mMol of fatty acid and 10 μL of hexadecane (as internal standard) were dissolved in 5 mL heptane in a 20-mL glass vial. Lipase preparation (500 mg) was added, the vial was sealed with a teflon-lined cap, and the suspension was shaken (95 rpm) at room temperature. Samples of 0.2 mL were subjected periodically to gas chromatographic analysis (column 1) and subsequent liquid-solid chromatography. Analogous experiments were carried out starting with ethyl octanoate and racemic 2-methylalkanoic acid as substrates.

Liquid-solid chromatography (LSC). The clear reaction mixture (0.2 mL) was placed on a water-cooled glass column (250 × 9 mm i.d.) filled with 5 g silica gel (Merck 7734, Merck, Darmstadt, Germany). Fraction I (pentane, 40 mL) eluted heptane and the hydrocarbon standard; fraction II (pentane/methylene chloride, 1:2) contained the esters; the free acids were eluted with 40 mL ether (fraction III). Fraction I was discarded; fractions II and III were concentrated to volumes of 0.2 mL by using a Vigreux column. Fraction III was used directly for derivatization and subsequent capillary gas chromatographic (GC) analysis (column 2). Fraction II was subjected to alkaline hydrolysis.

Alkaline hydrolysis. Methanolic potassium hydroxide solution (10%, 0.2mL) was added to the concentrated fraction II. The mixture was shaken at room temperature overnight. After addition of 1 mL 1N HCl the liberated acids were extracted with ether (3 × 20 mL). The ether extracts were dried over sodium sulfate, concentrated to a volume of 0.2 mL with a Vigreux column and subjected to derivatization and capillary GC analysis (column 2).

Derivatizations. After transfer of the samples to screw-capped vials and careful removal of the solvent by means of a nitrogen stream, the following procedures were applied: A (2-methylbutanoic acid), addition of 10 μL dry toluene and 5 μL (R)-(+)-1-phenylethylisocyanate, heating at 100°C for 12 hr, (18); B (2-methylhexanoic acid), addition of 1 μL acetyl chloride and 5 μL (S)-(+)-octan-2-ol, heating at 80°C for 15 hr. After addition of 0.4–1.0 mL of solvent (A, methylene chloride; B, ether) 1 μL of the mixture was used for capillary GC analysis on column 2.

Gas chromatography. Column 1: DB-Wax column (60 m × 0.32 mm i.d.; film thickness 0.25 μm, J&W, Folsom, CA), installed in a Carlo Erba Fractovap Series 2050 gas chromatograph (Carlo Erba, Milano, Italy) equipped with flame ionization detector (FID) and split injector (1:25); injector temperature, 200°C; detector temperature, 220°C; carrier gas, helium (2.5 mL/min); temperature program from 70 to 230°C at 4°C/min. Column 2: DB-210 column (60 m × 0.32 mm; film thickness 0.25 μm; J&W), installed in a Carlo Erba Fractovap 2900 gas chromatograph equipped with FID and split injector (1:20); injector temperature, 230°C, detector temperature, 250°C; carrier gas hydrogen (2.5 mL/ min). Separations of the diastereomeric derivatives were obtained as follows: (R)-1-phenylethyl-

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amides of (R,S)-2-methylbutanoic acid: column temperature, 135°C; retention times $t_{r(I)}$ [(R)-acid, (R)-isocyanate], 10.1 min; $t_{r(II)}$[(S)-acid and (R)-isocyanate], 10.6 min; (S)-2-octylesters of (R,S)-2-methylhexanoic acid: column temperature 90°C, retention times $t_{r(I)}$[(S)-acid, (S)-alcohol], 6.45 min; $t_{r(II)}$ [(R)-acid, (S)-alcohol], 6.7 min. The order of elution was determined by derivatization and GC analysis of optically enriched reference compounds (15).

Gas chromatography-mass spectrometry. A Finnigan MAT 4500 series (Finnigan MAT, San Jose, CA) quadrupole gas chromatograph-mass spectrometer coupled with an Incos data system was used. The fused silica column (DB-Wax) was inserted directly into the ion source. Ionization voltage, 70 eV; ion source temperature, 180°C; speed, 1 scan/s; mass range (*m/z*), 33–500.

RESULTS AND DISCUSSION

Reaction mixtures investigated in the present study consisted of heptane as solvent, containing racemic 2-methylalkanoates and fatty acids as substrates, hexadecane as internal standard and a lipase from *Candida cylindracea* (CCL) as biocatalyst. The course of the reaction was followed by means of periodic gas chromatographic/mass spectrometric analyses of aliquots. A separation of the reaction mixture according to the polarity of the components was achieved by liquid-solid chromatography on silica gel. The optical purities of product and remaining substrate were determined *via* capillary gas chromatographic separations of diastereomeric (R)-1-phenylethylamides (2-methylbutanoic acid) and (S)-2-octylesters (2-methylhexanoic acid), respectively.

Application of this procedure revealed that lipase from *Candida cylindracea* catalyzes the acidolysis of racemic 2-methylalkanoates with a preference for the (S)-configurated esters according to the reaction scheme in Figure 1.

Structural effects. The data obtained for various combinations of substrates are summarized in Table 1. Initial reaction rate and enantioselectivity (E), expressed as ratio

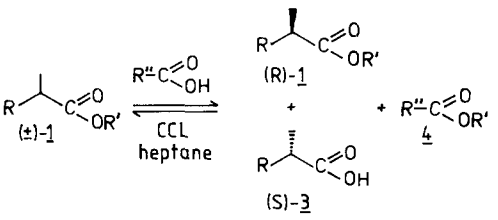

FIG. 1. Reaction scheme for the acidolysis of racemic 2-methylalkanoates catalyzed by lipase from *Candida cylindracea*.

of the specificity constants for the two enantiomers (19), are strongly influenced by the structures of the starting esters and acids, respectively.

In analogy to results obtained for CCL-catalyzed esterifications of 2-methylalkanoic acids (15), acidolyses of 2-methylbutanoates (entries 1a and 1b) proceeded faster than those of the corresponding 2-methylhexanoates, but with significantly lower enantioselection. A change of the alcohol moiety had only a slight impact on the stereochemical outcome of the reaction.

On the other hand, both enantioselectivity and velocity of the acidolysis of 2-methylhexanoates are markedly influenced by changes of the alcohol chain of the starting ester (entries 2a–2e). Comparable to strategies applied for transesterifications *via* alcoholysis (20), the use of an activated ester with a good leaving group (trifluoroethanol) leads to maximum reaction rate. However, in the present case, enhanced enantioselection (at the expense of the reaction velocity) could only be achieved by increasing the hydrophobic character of the alcohol moiety of the ester substrate.

Variation of the acid substrate (entries 3a–3e) had only a slight effect on the enantioselectivity. However, the choice of the acid is important in terms of reaction rate—acids with aromatic structures may not be accepted by the enzyme; the best results were obtained with oleic acid,

TABLE 1

CCL-Catalyzed Acidolysis of Racemic 2-Methylalkanoates *via* the Reaction Scheme in Figure 1

Ester (±)-1 RCH(CH_3)COOR′	Acid 2	t (hr)	Conversion (%)	Relative rate (%)[b]	(R)-1 e.e.(%)	(S)-3 e.e.(%)	E[a]
1a C_2H_5 ethyl	Octanoic	2	9	68	6.2	64.5	6
1b C_2H_5 octyl	Octanoic	2	7	56	5.9	72.3	7
2a C_4H_9 ethyl	Octanoic	23	24	16	25.6	81.8	13
2b C_4H_9 trifluoroethyl	Octanoic	3	20	100	21.2	84.4	15
2c C_4H_9 cyclohexyl	Octanoic	21	8	6	8.2	88.1	17
2d C_4H_9 octyl	Octanoic	20	15	11	17.1	95.6	52
2e C_4H_9 octadecyl	Octanoic	70	23	5	29.6	97.6	126
3a C_4H_9 ethyl	Cyclohexanoic	48	24	7	22.7	73.1	8
3b C_4H_9 ethyl	Phenylacetic	70	3	0.6	2.7	85.2	13
3c C_4H_9 ethyl	Dodecanoic	8	14	26	13.8	84.8	14
3d C_4H_9 ethyl	Octadecanoic	23	25	17	27.2	81.6	13
3e C_4H_9 ethyl	Oleic	8	16	30	15.7	82.4	12
4a C_4H_9 octyl	Oleic	14	21	22	25.1	94.9	49
4b C_4H_9 octadecyl	Oleic	72	32	7	46.5	97.1	106

[a] Reference 19.

[b] Reaction rate determined for entry 2b: v = 0.067 $\mu Mol \cdot h^{-1} \cdot mg^{-1}$ CCL.

TABLE 2

CCL-Catalyzed Acidolysis Starting from Ethyl Octanoate and Racemic 2-Methylalkanoic Acids

Acid	t (hr)	Conversion (%)	(R)-2-Methyl-alkanoic acid e.e.(%)	Ethyl (S)-2-methylalkanoate e.e (%)	E
(±)-2-Methylbutanoic	2	17	7.8	39.0	3
(±)-2-Methylhexanoic	8	9	6.6	70.0	6

a fatty acid found in many natural substrates of lipases. Further systematic studies with acids and esters of varying structures might offer interesting information regarding the binding site of the enzyme.

Modified approach. A kinetic resolution of 2-methylalkanoic acid enantiomers by means of acidolysis could also be achieved by starting with the racemic acid rather than an ester substrate. As shown in Table 2 for the reaction of 2-methylalkanoic acids with ethyl octanoate, the lipase retains its preference for the (S)-enantiomer, thus leading to product and remaining substrate with configurations opposite to those obtained by the scheme shown in Figure 1. However, a comparison of the data in Table 2 with entries 1a and 2a in Table 1 demonstrates that this modification of the approach reduces the enantioselectivity of the reaction.

Thermodynamic parameters. For lipase-catalyzed acidolyses of triacylglycerols, incorporation rates of fatty acids remained below the theoretical values, and thereby indicated the existence of an equilibrium at the late stage of the reaction (21). In order to assess this phenomenon in the acidolysis of chiral 2-methylalkanoates, investigations of some of the substrate combinations listed in Table 1 were extended beyond the initial reaction stages.

Following the time course of the acyl exchange between octyl 2-methylhexanoate and oleic acid (Fig. 2) revealed that this process is also characterized by the presence of an equilibrium stage after about 40% conversion. Figure 3 displays the absolute amounts of the different enantiomers, calculated from quantitative values and enantiomeric compositions at each time of analysis. The resulting time course is typical for enzyme-catalyzed kinetic resolution in a reversible system (22)—the preferred reaction of the (S)-ester in the initial stage approaches an equilibrium. The equilibrium constant can be calculated from the concentrations of remaining substrate and product of the faster reacting enantiomer at this equilibrium stage. In the present example (Fig. 3), a constant (K = 0.33) is obtained from the quotient of the equilibrium concentration of octyl (S)-2-methylhexanoate (0.06 mMol) and (S)-2-methylhexanoic acid (0.18 mMol). The ongoing reaction of the "slower" (R)-enantiomer results in decreasing optical purities of both product and remaining substrate.

If biocatalyzed kinetic resolutions of enantiomers proceed *via* reversible processes, the obtainable optical purities of product and remaining substrate are determined not only by a kinetic parameter, the enantioselectivity (E) of the enzyme, but also by a thermodynamic function, the equilibrium constant (K) of the reaction (22,23). Therefore,

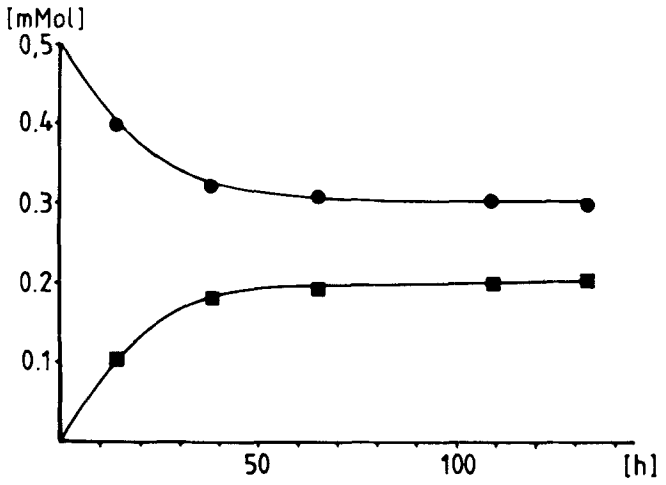

FIG. 2. Time course of the CCL-catalyzed reaction between octyl 2-methylhexanoate and oleic acid. ●, Octyl 2-methylhexanoate; and ■, 2-methylhexanoic acid (ethyl oleate).

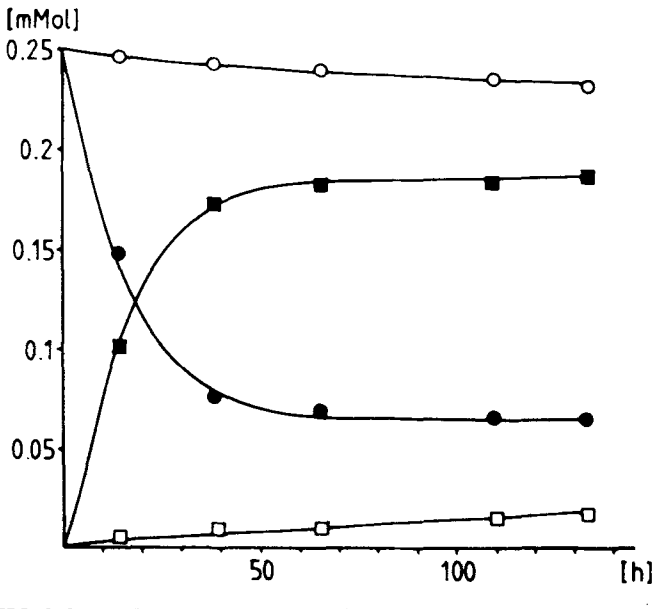

FIG. 3. Stereochemical course of the CCL-catalyzed reaction between octyl 2-methylhexanoate and oleic acid. ●, Octyl (S)-2-methylhexanoate; ○, Octyl (R)-2-methylhexanoate; ■, (S)-2-methylhexanoic acid; and □, (R)-2-methylhexanoic acid.

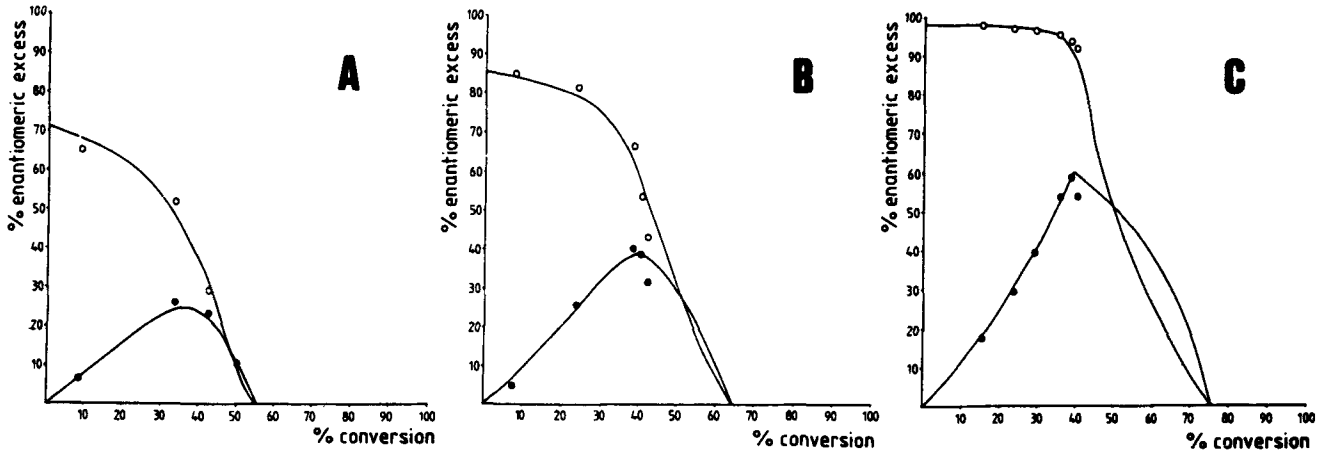

FIG. 4. Graphs expressing the relationship between conversion rate and enantiomeric excesses of product (e.e.$_P$) and substrate (e.e.$_S$) for CCL-catalyzed acidolyses between octanoic acid and A, ethyl 2-methylbutanoate (E=6; K=0.8); B, ethyl 2-methylhexanoate (E=13; K=0.6); and C, octadecyl 2-methylhexanoate (E=126; K=0.32). ○, Experimentally determined values for e.e.$_P$; and ●, experimentally determined values for e.e.$_S$. As confirmed in a personal communication by Professor Sih, the generation of these graphs cannot be based on the equations presented in footnote 10 of reference 22. The correct equations to be applied are as follows: $c = (2\text{-}x\text{-}x^E)/[2(1+K)]$, $ee_P = (x\text{-}x^E)/(2\text{-}x\text{-}x^E)$, $ee_S = (x\text{-}x^E)/(2K+x+x^E)$ for $0 \leq x \leq 1$.

equations valid only for quantitative treatment of irreversible kinetic resolutions (19) cannot be applied. Expressions relating the enantiomeric excesses of product and substrate as a function of the conversion rate at different values of both E and K have been developed (22). Figure 4 presents such theoretical curves calculated (see note in caption of Fig. 4) on the basis of the experimentally determined values of E and K for three different substrate combinations. The good agreement between the actual enantiomeric compositions in the course of the reaction and these graphs demonstrates the successful simulation of the resolution process by the kinetic model. Comparable to studies of enzymatic resolutions of chiral alcohols *via* esterifications and transesterifications, respectively (23), significant deviations were only observed after the reactions had reached their equilibrium.

The reaction profiles presented in Figure 4 impressively demonstrate the influence of enantioselectivity and equilibrium constant on scope and limitations of the CCL-catalyzed acidolysis of chiral 2-methylalkanoates. The initially obtainable enantiomeric excess of the product and the time when the reaction has to be stopped to assure maximum optical purity of the substrate are governed by these parameters, which can be influenced by changing the structures of the starting substrates. Increasing the chainlength of the alcohol moiety of the ester is favorable for both the enantioselectivity and the equilibrium of the reaction.

Potential optimization. A comparison of the reaction rates of CCL-catalyzed acidolyses of 2-methylalkanoates with those observed for esterifications of 2-methylalkanoic acids under similar conditions (15) confirms previously reported results; lipase-catalyzed acidolyses proceed slower than the corresponding esterifications (7,24,25).

In the present study, enzyme and solvent were employed "straight from the bottle". The mechanism generally accepted for lipase-catalyzed acidolysis involves hydrolysis of the ester followed by reesterification of the liberated alcohol moiety (26); water plays an important role in this process (25). Therefore, careful adjustment of the optimum water content of the reaction medium (25,27) may be favorable. The amount of lipase needed should be optimized, the biocatalyst could be adsorbed or immobilized on an adequate support (28,29), and the ester could be used in excess to suppress the reverse reaction. Variations of these parameters might lead to reaction rates and product yields suitable for kinetic resolutions of the enantiomers of 2-methylalkanoates *via* acidolysis at large-scale.

ACKNOWLEDGMENT

The author thanks Mrs. Irmgard Roling for skillful technical assistance.

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[Received June 4, 1991; accepted September 24, 1991]