Effects of Substrate Structure on Lipase-Catalyzed Transesterification of ω-Substituted 1-Alkanols in Organic Solvents

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Lipase-catalyzed transesterifications of ω-phenyl-1-alkanols with vinyl acetate in organic solvents have been investigated. Benzyl alcohol is the most reactive substrate among those studied. It is proposed that a lipase form *Pseudomonas cepacia* (PCL) has a domain to attract an aromatic moiety of a substrate.

Lipases are useful biocatalysts for organic chemists in the sense that they can maintain enzymatic activity not only in aqueous solutions but also in organic media. Indeed, in an organic solvent a lipase catalyzes esterification, transesterification, or even aminolysis.[1—3] Thus, many chiral alcohols and esters that have important synthetic potentials have been synthesized frequently by using lipase-catalyzed transesterifications.[4] However, there have been little information on mechanisms and substrate selectivity of a lipase in organic media. The latter is particularly useful and important when using the lipase-catalyzed reaction for kinetic resolution of racemic alcohols and acids. Several reports have contributed to understanding the effects of chain length in alcoholic and carboxylic substrates on the stereoselectivity and catalytic efficiency of a lipase in organic media.[5—13] However, since only scattered results on limited substrates are available from the literature, it is difficult to understand the effects of carbon-chain length of the substrate on reactivity and selectivity of the lipase reaction systematically. In this paper, therefore, we report our results from a systematic study on substrate selectivity of a lipase from *Pseudomonas cepacia* (PCL). ω-Phenyl-1-alkanols were used as substrates for transesterification with vinyl acetate. ω-Cyclohexyl-1-alkanols were also studied to discover the role of phenyl substituents unambiguously. An immobilized lipase was used throughout this study. Details of immobilization and amounts of lipase are described in the Experimental section. The substrates used for the reaction are listed in Scheme 1.

Results

Kinetics, analyzed as pseudo-first order in alcohol, were followed by observing the increase in concentration of produced acetyl esters.

To discuss the reactivity of alcohol from the viewpoint of kinetics with each alcohol, it is necessary to secure constancy in the concentration of acyl lipase. Thus, we first studied K_m for vinyl acetate in the reaction with 2-phenylethanol. A large K_m (more than 1 M) was obtained. Since 500 mM (1 M=1 mol dm^{-3}) concentration of vinyl acetate was used throughout this study, it should be noted that the reaction rates were not maximum in the sense that not all the lipases were acylated. The situation will be discussed later. Second, initial rates for acylation of ω-phenylalkanols (**1a**—**5a**) and ω-cyclohexyl-1-alkanols (**1b**—**4b**) were measured and the influence of the number of methylene groups between hydroxyl group and ω-substituent was investigated. The results are summarized in Table 1. Kinetic parameters for all the substrates studied are summarized in Table 2. The Hanes–Hoolf plot[14] was used for calculating K_m and V_{max} of the reaction with each alcohol. Here, the value of V_{max} is normalized as V_{max}/mg-lipase.

Discussion

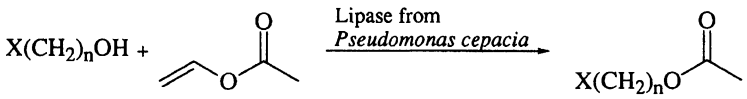
Acylation of a lipase with vinyl acetate is usually an initial process.[15] In addition, since each substrate experimentally exerts its own characteristic kinetic parameters, we are convinced that the kinetic parameters obtained reflect the selectivity of acylated lipase for the substrate alcohols.

Although it is ideal to run the reaction with such a high concentration of vinyl acetate that the rate becomes independent of its concentration, unreasonably high concentrations of a reagent may change the properties of the reaction medium, and, at the same time, a large K_m (2.61 M in benzene and 1.64 M in diisopropyl ether) inevitably predicts that the reaction rate is not strictly sensitive to the concentration of vinyl acetate. Therefore, the reaction run with 500 mM vinyl acetate may be accepted safely as one independent of the con-

Table 1. Initial Rates for the Lipase-Catalyzed Transesterification

Substrate	Initial rate/μM s^{-1} mg^{-1} lipase	
	Benzene	DIE[a]
1a	8.87	18.9
2a	1.21	1.92
3a	4.81	4.71
4a	3.48	5.09
5a	3.56	5.49
1b	1.45	4.08
2b	1.76	2.00
3b	1.83	2.68
4b	3.54	5.45

a) Diisopropyl ether.



1a : X = Phenyl, n = 1 **1b** : X = Cyclohexyl, n = 1
2a : X = Phenyl, n = 2 **2b** : X = Cyclohexyl, n = 2
3a : X = Phenyl, n = 3 **3b** : X = Cyclohexyl, n = 3
4a : X = Phenyl, n = 4 **4b** : X = Cyclohexyl, n = 4
5a : X = Phenyl, n = 5

Scheme 1.

Table 2. Kinetic Parameters for the Lipase-Catalyzed Transesterification

Substrate	K_m/mM		V_{max}/μM s^{-1} mg^{-1} lipase		$10^4 V_{max}/K_m$/s^{-1} mg^{-1} lipase	
	Benzene	DIE[a)]	Benzene	DIE[a)]	Benzene	DIE[a)]
1a	3.55± 1.09	7.14± 1.49	10.7 ± 0.5	25.8± 1.2	30.2 ± 9.2	36.1 ± 7.4
2a	31.6 ± 3.6	97.0 ± 3.6	3.13± 0.19	10.5± 0.2	0.988± 0.102	1.08± 0.03
3a	34.1 ± 2.7	60.4 ± 5.2	13.0 ± 0.4	19.3± 0.6	3.83 ± 0.28	3.19± 0.26
4a	43.2 ± 3.8	38.1 ± 3.8	11.0 ± 0.5	14.8± 0.5	3.89 ± 0.36	2.55± 0.21
5a	26.4 ± 3.6	43.4 ± 2.8	8.27± 0.35	17.5± 0.6	3.13 ± 0.43	4.04± 0.23

a) Diisopropyl ether.

centration of vinyl acetate, although this concentration of vinyl acetate does not constitute the V_{max}. Consequently, it should be noted that kinetic parameters are valuable only when they are discussed in relative magnitude.

Since vinyl acetate is transformed into acetaldehyde as the result of initial formation of acylated lipase, the sole nucleophile which can attack the acylated lipase in any instant of the reaction is the substrate alcohol only. Thus, the use of vinyl acetate is an ideal device to eliminate undesired reverse reactions to decompose the product acetate.[16)]

Table 1 shows that initial rate of **1a** is extraordinarily larger than the others and that of **2a** is much smaller than those of its higher analogs. An odd/even alternating effect is observed in benzene.

Since enzyme kinetics are composed of plural parameters, it is necessary to study individual parameters to discuss the mechanism of the reaction in detail. Thus, the results summarized in Table 2 contribute to elucidate a major factor for the observed tendency in reactivity.

When K_m is inspected, it is recognized that the value for **1a** is extraordinarily smaller than those for others. The value of **2a** is 9 times as large as that of **1a** in benzene and it is 14 times in diisopropyl ether.

The observation can well be understood by assuming an appropriate domain for attracting a phenyl group in the vicinity of the reacting center of the lipase (anchor effect). Since the phenyl group in **1a** fits in the domain, this substrate can undergo the reaction easily in both benzene and diisopropyl ether; K_ms for **1a** in these two solvents do not differ appreciably. On the contrary, K_m for **2a** is larger and V_{max} is smaller than those of others. The phenyl group of **2a** incorporated into the lipase has to be deviated from the phenyl-attracting domain to set its hydroxyl group at an appropriate position for the reaction. However, at the same time in this case, the phenyl group is attracted to the domain. Thus, when **2a** is incorporated into the lipase, the phenyl group tends to approach the domain to set the hydroxyl group at an appropriate position for the reaction. In other words, there is a competition for phenyl and hydroxyl groups to reside in appropriate positions. Because there are only two methylene groups between the phenyl and the hydroxyl groups, **2a** cannot attain the best-fit conditions, and the "disordered" ES complex forms. When the carbon-chain becomes longer, steric requirement to set both phenyl and hydroxyl groups at suitable positions may be reduced owing to the flexibility of the carbon chain, or the competition becomes less important.

The proposal of phenyl domain predicts that K_m in benzene might become larger due to competitive inhibition by the solvent molecule. However, it is recognized, by introduction of the concept of "induced-fit",[17)] that the expectation is not necessarily true. The benzene molecule, which has not polar side chain, cannot be an effective competitive inhibitor.

The chemical reactivity of an alcohol trapped in the pocket at active site of the lipase is reflected more precisely in a parameter V_{max}/K_m. The difference in reactivity of the phenyl-substituted substrates in benzene and diisopropyl ether is not significant for all the substrates studied when their V_{max}/K_m values are compared. Although the difference in V_{max}/K_m between these two solvents is a little bit larger than experimental error, discussion of such a small difference may be meaningless except for **1a**. Similarity in reactivity may stem from similarity in hydrophobicity of these two solvents.[18)] It has been claimed that hydrophobicity of the solvent primarily is important for determining the

reactivity.[19] V_{max}/K_m values indeed show that the reactivities of alcohols are almost equal for all the substrates except for **1a**. Non-specified interaction between the solvent and lipase holds the conformation of the enzyme so as to result in about twice more favorable K_m and disfavorable V_{max} in benzene in comparison to those, respectively, in diisopropyl ether.[20] The composite effect of K_m and V_{max} are cancelled in V_{max}/K_m or in the initial rate affording solvent-independent kinetics in these solvents. The observation confirms the idea that the hydroxyl group in the substrate is set at the reaction center correctly, or at least at the vicinity of correct position, regardless of the length of carbon chain when the substrate is subjected to the reaction. Otherwise, no chemical reaction proceeds and we cannot recognize the formation of such an abortive complex as those mentioned above.

The phenyl group in the substrate was replaced by a cyclohexyl group to test the above-mentioned idea that the binding site of the lipase has a special affinity for an aromatic moiety. Reactivities of substrates with a ω-cyclohexyl group, **1b**—**4b**, in benzene are similar. In other words, **1b** is not a special substrate as **1a** is and behaves similarly to others. In diisopropyl ether, **2b** has a smaller reactivity than that of **1b**, then the reactivity increases with the increase in carbon-chain length. The results suggest that the phenyl-attracting domain can accept a cyclohexyl group by its hydrophobic character though the attracting force is very weak compared to that for a phenyl group. Here again, the alcohol with $n=2$ might be disturbed in its orientation in the pocket.

Combining the observations for substrates **1**—**5**, we propose that, once a substrate is incorporated into the pocket, the substrate finds a domain to attract the aromatic part. Location of the domain is quite close to the active site so that benzyl alcohol, **1a**, becomes the best substrate among those studied. The cyclohexyl moiety is also attracted to the domain by hydrophobic interaction although the force is not so strong as that for a phenyl group.

Kinetic parameters for a lipase-catalyzed esterification of a series of long-chain alkanols with no ω-substituent have been reported.[10] Here, little changes in K_m and V_{max} have been recognized. Dordick et al. reported catalytic activity and stereoselectivity of a lipase from *Candida rugosa* for esterification of an alcohol with various carboxylic acids as the acyl donor.[21,22] The carboxylic acids of even-numbered carbon chain exerted a V-shape correlation for reactivity. However, since the lipase from *C. rugosa* is claimed to be composed of several proteins that exert lipase activity[23] in contrast to a lipase from *P. cepacia* which has been used for this study, their results cannot be compared with ours directly.

Morgan et al. have reported that an alcohol substituted by a phenyl group at its α-position affords the corresponding ester with large enantioselectivity in transesterification catalyzed by porcine pancreatic lipase (PPL).[24] Although the origins of their and our lipases differ largely, it is easily conceivable that their lipase also has a domain for recognition of unsaturation.

X-Ray crystallographic analysis of lipases from *Candida rugosa*[25,26] and *Rhizomucor miehei*[27] have been reported. It is known that these enzymes have hydrophobic regions lined with phenyl rings. This makes it possible to expect that a π–π stacking interaction between one of aromatic residues in the enzyme and a phenyl group in substrate seems to be an important factor to recognize and anchor the aromatic substrate, preferentially resulting in a small K_m.

Experimental

Instrumental. Gas chromatograms were recorded on a Shimadzu GC-14A gas chromatograph.

Materials. Substrates and organic solvents were obtained from Nacalai Tesque Co., Tokyo Kasei Co., Wako Jun-yaku Co., and Aldrich Chemical Co. Solvents were dried before use over 4 Å molecular sieves (Linde) or CaH_2. Celite was purchased from Nacalai Tesque Co. All substrates for reaction gave satisfactory results in elemental analyses and ^{1}H NMR spectroscopy.

Preparation of ω-Phenyl and ω-Cyclohexyl-1-alkyl Acetate. In general, 7 mmol of acetyl chloride was added to 20 ml of a benzene solution containing 2.5 mmol of ω-phenyl or ω-cyclohexyl-1-alkanol and 13 mmol of dry pyridine. The mixture was stirred overnight at room temperature and filtered. The filtrate was washed with 0.1 M hydrochloric acid, saturated solution of sodium hydrogen carbonate, and that of sodium chloride in this order, then dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was chromatographed on a silica gel column using hexane–ethyl acetate (2 : 1) as an eluent. The combined fraction was distilled to give a ω-phenyl or ω-cyclohexyl-1-alkyl acetate in about 40% yield.

Immobilization of Lipase. As a typical procedure, a lipase from *Pseudomonas cepacia*, Amano PS (10 mg), and Celite 353 (990 mg) were mixed in 4 ml of distilled water. The mixture was dried under reduced pressure. This immobilized lipase is abbreviated as 1/100-PS in this paper. In a similar manner as described above, 34 mg of the lipase was immobilized with 986 mg of Celite. This preparation is abbreviated as 1/30-PS in this paper. The catalytic activity per milligram of the lipase contained in 1/100-PS was different from that of native lipase in diisopropyl ether as the reaction medium. When 2-phenylethanol was used as a substrate for the reaction in diisopropyl ether, the initial rates per milligram of a native lipase were 3.64 and 4.84 $\mu M\,s^{-1}\,mg^{-1}$ lipase for 1/30-PS and 1/100-PS respectively. Consequently, initial rates measured for 1/100-PS in diisopropyl ether were multiplied by 0.75 for normalization to elucidate K_m and V_{max}. The normalization factor for 1/100-PS against the native lipase in benzene was 0.74. Similarly, initial rates observed for 1/30-PS in benzene were multiplied by 0.30.

Product Analyses. Conditions for gas chromatographic analyses are as follows. **1a**; OV-1701 Bonded, 30 m, 145 °C: **2a**; OV-17101 Bonded, 30 m, 160 °C: **3a**; OV-101 Bonded, 25 m, 170 °C: **4a**; OV-1701 Bonded, 30 m, 175 °C:

5a; OV-101 Bonded, 25 m, 170 °C: **1b**; OV-1701 Bonded, 30 m, 130 °C: **2b**; OV-1701 Bonded, 30 m, 140 °C: **3b**; OV-1701 Bonded, 30 m, 155 °C: **4b**; OV-1701 Bonded, 30 m, 170 °C. Under these conditions, the product peak on the chromatogram coincided with that of the acetyl ester prepared as an authentic sample. No other peaks except for that of the starting alkanol was detected on the chromatogram and the increase in the concentration of product agreed directly with the decrease in the concentration of the starting alkanol. The fact predicts that the transesterification is free from side reactions.

Kinetics for Lipase-Catalyzed Transesterification. An immobilized lipase was placed in a vial and 2 ml of a solvent containing 500 mM vinyl acetate, 20 mM alkanol, and a higher hydrocarbon (tridecane, tetradecane, pentadecane) as an internal standard for gas chromatography was added to the vial. Then, the resulting suspension was stirred magnetically at 35 °C. Periodically, samples were withdrawn and analyzed on gas chromatography. Initial rate was measured by following the increase in the concentration of the product, acetyl ester. Kinetics were followed up to about 20% conversion of the substrate and five to seven data points were usually collected. Kinetic parameters for transesterification were measured with immobilized lipase suspended in 2 ml of an organic solvent containing 500 mM vinyl acetate. Initial alkanol concentration was changed from 6 to 280 mM. Initial rates were measured by the aid of gas chromatography as described above. The Hanes–Hoolf ($[S]/\nu_0$ vs. $[S]$) plot[14] was used for elucidating K_m and V_{max}.

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