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Factors Influencing Enantioselectivity of Lipase-Catalyzed Hydrolysis

Masamichi Kinoshita and Atsuyoshi Ohno*

Institute for Chemical Research, Kyoto University
Uji, Kyoto 611, Japan

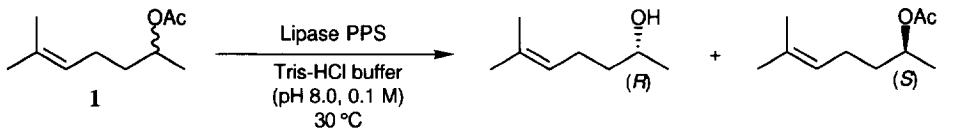
Abstract: Some factors influencing the enantioselectivity of lipase-catalyzed hydrolysis have been investigated. Enantioselectivity of the reaction increases with the increase in the concentration of substrate. Addition of certain organic solvent to the reaction mixture results in the change in enantioselectivity: hexane, cyclopentane, benzene and diisopropyl ether improve the enantioselectivity, whereas cyclohexane deteriorates it. A detergent, Triton X-100, and 2,2-dimethylbutane do not influence the enantioselectivity under the condition that kinetics is saturated with respect to the substrate. The fluctuation of enantioselectivity depends on the binding of hydrophobic compound to the lipase.

INTRODUCTION

Organic solvent affects activity and stereoselectivity of enzymatic reactions in various ways.[1] Although there is certainly a tendency for the activity to increase with the increase in hydrophobicity of solvents,[2] no general rule has been found between the stereoselectivity and solvent properties. We have recently proposed that a solvent molecule(s) is incorporated into the substrate-binding pocket controlling the enantioselectivity of lipase; a solvent affects the enantioselectivity of lipase as a reagent rather than a medium.[3] In this paper, we would like to propose the role of organic solvent in molecular level on the enantioselectivity of lipase-catalyzed hydrolytic reaction.

RESULTS AND DISCUSSION

In a previous paper from our laboratory, we focused the attention on the effect of solvent in the lipase-catalyzed transesterification of a racemic mixture of 6-methyl-5-hepten-2-ol (sulcatol) with vinyl acetate.[3] In this paper, we are concerned with factors influencing enantioselectivity of lipase-catalyzed hydrolysis. Acetate of sulcatol (2-acetoxy-6-methyl-5-heptene; **1**) was employed as a substrate for the reaction in Tris-HCl buffer (pH 8.0, 0.1 M). Sulcatol is used as an important chiral building block to synthesize bioactive natural products such as (*R*, *R*)-(-)-pyrenophorin.[4] The reaction is illustrated in Scheme 1.



Scheme 1

Amano PS is a lipase immobilized on celite. The immobilized lipase may partly separate from celite and dissolve into water, which causes the difficulty in analyzing the reaction. Therefore, we used the purified lipase, PPS (Purified PS; 2,200,000 unit/g, unimmobilized) instead of PS.

Effect of Substrate Concentration

Chemists generally tend to keep reaction system homogeneous to enhance the efficiency of reaction and a co-solvent such as acetone is often employed to dissolve hydrophobic substrate in enzymatic hydrolytic reactions.[5,6,7] However, we think that, in lipase-catalyzed hydrolysis, a co-solvent for this purpose is not necessarily required when the substrate is liquid, because a lipase is activated at the interface between water and lipid (*interfacial activation*).[8] The activation depends on the area of the interface; larger the area, higher the activation becomes. Furthermore, the area increases with the increase in the concentration of lipid. The substrate, **1**, is not lipid, but it is hydrophobic enough to form the interface, so we investigated the effect of *concentration*[9] of the substrate on the activity and enantioselectivity of the lipase. The results are summarized in Table 1.

Table 1. Dependence of Activity and Enantioselectivity on Concentration of Substrate in the Lipase-Catalyzed Hydrolysis.

$[E]_0$[a] (unit/ml)	$[S]_0$[b] (mmol/l)	$[S]_0/[E]_0$ (μmol/unit)	10^2k_R[c] (h^{-1})	10^3k_S[c] (h^{-1})	E-value
17.6	0.784	0.0445	11.9±1.9	15.0±0.8	7.9±0.4
17.6	2.35	0.134	15.5±0.3	14.1±0.8	10.9±0.3
17.6	5.88	0.334	5.3±0.3	4.0±0.2	13.2±0.3
17.6	8.47	0.481	3.8±0.3	2.7±0.2	13.8±0.3
17.6	11.8	0.670	2.57±0.12	2.05±0.12	12.5±0.4
88.0	2.35	0.0267	11.6±1.8	29.9±4.1	3.9±0.1
88.0	5.88	0.0668	16.9±1.5	19.5±1.5	8.7±0.1
88.0	8.40	0.0955	16.5±2.5	15.5±0.5	10.6±1.2
88.0	11.8	0.134	12.9±1.3	11.6±0.5	11.1±0.6
88.0	14.7	0.167	13.2±0.7	11.7±<0.1	11.3±0.5

(a) Concentration of lipase PPS.

(b) Concentration of racemic 6-acetoxy-5-methyl-2-heptene (**1**).

(c) Rate constants for the (*R*)- and (*S*)-**1**, respectively.

Although the present reaction system is heterogeneous due to hydrophobic substrate, the kinetics appears to be first-order similarly to the lipase-catalyzed transesterification in organic solvents.[3] An example is depicted in Fig. 1.

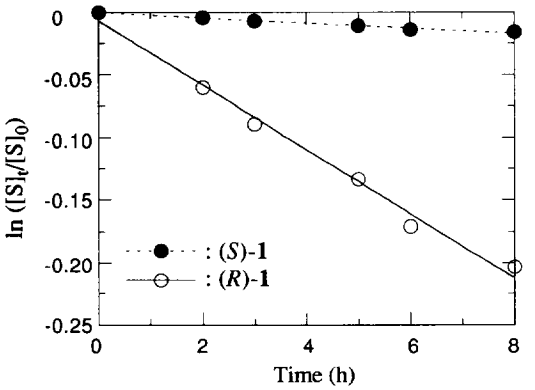


Figure 1. Kinetic plots for the lipase-catalyzed hydrolyses of racemic **1**. $[S]_0$; initial concentration, $[S]_t$; concentration of the (*R*)- or (*S*)-**1** after an appropriate time interval. Condition: $[E]_0$ = 17.6 unit/ml, $[S]_0$ = 11.8 mmol/l.

Therefore, we elucidated the rate constants for the (*R*)- and (*S*)-**1** (k_R and k_S, respectively) and E-values based on the same method as reported in the previous paper.[3] It is well-known that kinetics of a lipase-catalyzed hydrolysis does not follow simple Michaelis-Menten equation, $v = k_{cat}[E]_0[S]/(K_m+[S])$.[10] In the present study, the dependence of reaction rate per concentration of lipase on the concentration of the substrate appears very characteristic. A plot is depicted for the reaction rate with (*R*)-**1** in Fig. 2*a*.

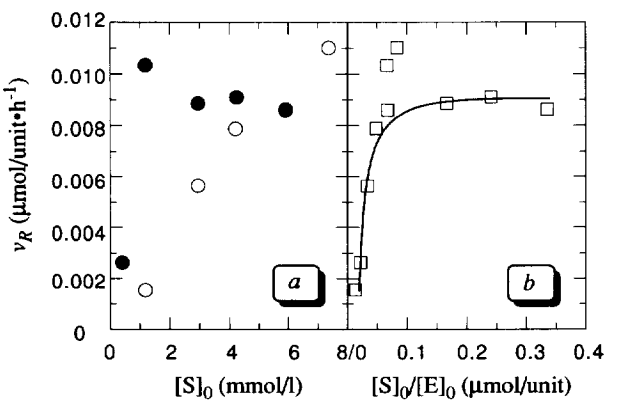


Figure 2. The rates of (*R*)-**1** measured at low and high concentrations of lipase. v_R is defined as the product of k_R and $[S]_0/[E]_0$, and it means the rate per concentration of lipase. $[S]_0$ is the concentration of (*R*)-**1** (Note: This is half of $[S]_0$ in Table 1). $[E]_0$: ○, 88.0; ●, 17.6 (unit/ml).

If this reaction follows Michaelis-Menten equation, two series of the rates should be superimposable on the same curve in Fig. 2*a*, because the reaction rate is proportional to the concentration of enzyme. Instead, Fig. 2*a* suggests that lipase is activated when its concentration is low. The result is abnormal from the viewpoint of enzymatic reactions, but we have a key to resolve this riddle. When $[S]_0/[E]_0$ instead of $[S]_0$ is employed for the abscissa, an abrupt saturation curve which is different from that proposed by the Michaelis-Menten equation appears (Fig. 2*b*), which means that the activity of the lipase is controlled by the concentration of the substrate interacting with the lipase. Since hydrophobic surface of the substrate activates the lipase, the reaction rate increases dramatically with the increase in the concentration of substrate. Furthermore, the lipase hydrolyzes the substrate at a certain rate after the substrate is saturated for the lipase.

It is worthy of note that the E-value increases with the increase in the concentration of substrate. By definition, E-value should not be affected by the concentration of a substrate.[11] However, if the concentration of an enzyme is larger than that of a racemic substrate and the enzyme is not so enantioselective to the substrate, E-value may depend on the concentration of substrate, because the competitions between the (*R*)- and (*S*)-enantiomers may not occur fully. In the present reaction, the initial concentration of the lipase ($[E]_0$) is at least 1000 times as little as the concentration of substrate ($[S]_0$), so the concentration of the lipase acting at the interface is less than 0.1 percent of the concentration of substrate molecule. Thus, we can discard the possibility. The remaining candidate is the change in conformation of the lipase.

It is well-known that a lipase has a lid covering the active site, which is opened to a solvent only when the lipase interacts with the lipid-water interface.[12] Furthermore, the movement of the lid changes the orientation of amino acid residues that construct a part of the active site such as oxyanion hole.[13] It has also been reported that this conformational change is related with the enantioselectivities in the hydrolytic reactions catalyzed by *Humicola lanuginosa* and *Rhizomucor miehei* lipases in water.[14,15]

In the present reaction, the enantioselectivity is beautifully correlated with $[S]_0/[E]_0$, similarly to the reaction rates, which is depicted in Fig. 3.

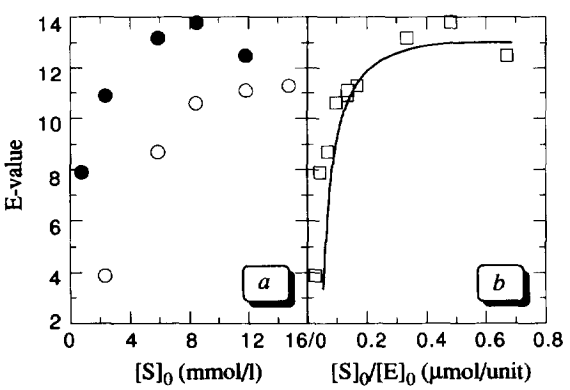


Figure 3. Dependence of enantioselectivity in the lipase-catalyzed hydrolysis on the concentration of racemic **1**, $[S]_0$. $[E]_0$: ○, 88.0; ●, 17.6 (unit/ml).

The observation means that the enantioselectivity of the lipase also depends on the concentration of the substrate interacting with the lipase. The interaction site may be a lid. Although three-dimensional structure of lipase PS (from *Pseudomonas cepacia*) has not yet been elucidated, it is plausible to assume the existence of a lid in PS based on the structure of *Pseudomonas glumae* lipase which has a lid and has high amino-acid sequence identity (84 %) to PS.[16,17] With the assumption that the open-lid lipase has higher enantioselectivity than the closed-lid one, the present result can be understood easily.

Effect of Organic Solvents Added to the Reaction System

In order to find more effective compounds to improve the activity and enantioselectivity of the lipase than **1**, we investigated the effect of organic compounds as addenda, which are regarded as solvents in common, to the reaction system for the lipase-catalyzed hydrolysis. They have been employed as solvents in the previous investigations.[3] The reactions were carried out under the conditions that the substrate and the addendum were well dispersed in a reaction vial.[18] The results are summarized in Table 2.

Table 2. Effect of Addenda on the Lipase-Catalyzed Hydrolysis.[a]

Addendum[b]	10^2k_R (h^{-1})	10^3k_S (h^{-1})	E-value
None	2.57±0.12	2.05±0.12	12.5±0.4
Hexane	1.89±0.05	0.83±0.01	22.8±0.6
DMB[c]	3.52±0.15	2.67±0.22	12.9±0.6
Cyclopentane	2.91±0.09	1.16±0.08	24.8±1.4
Cyclohexane	2.01±0.09	2.22±0.11	9.0±0.2
Benzene	0.67±0.05	0.21±0.03	31.1±3.3
Diisopropyl ether	2.92±0.08	1.47±0.07	19.7±0.6

(a) Condition: $[E]_0$ = 17.6 unit/ml, $[S]_0$ = 11.8 mmol/l.

(b) All compounds are added by 16 mg/ml.[18]

(c) 2,2-Dimethylbutane.

Hexane retards the hydrolyses of both (*R*)- and (*S*)-**1** with enhancement of the enantioselectivity; hexane is an (*S*)-selective inhibitor. On the other hand, 2,2-dimethylbutane (DMB) activates the hydrolyses of both (*R*)- and (*S*)-**1**, but it does not affect the enantioselectivity; DMB is a nonselective activator. Thus, these two compounds afford entirely different results to the lipase despite the fact that these compounds are similar in properties *as solvents*, such as polarity (ε) and hydrophobicity (log*P*).

Cyclopentane enhances (*R*)-activity[19] and reduces (*S*)-activity[19]. As a result, the enantioselectivity is improved largely. Cyclohexane, on the other hand, behaves opposite to cyclopentane resulting in the decrease in enantioselectivity. Thus, a small difference in ring size yields entirely different result in the activity of a lipase.

Benzene is a stronger and more (*S*)-selective inhibitor than hexane. On the other hand, diisopropyl ether acts

on the lipase in the same manner as cyclopentane, although the hydrophobicity of the former is equivalent to that of benzene. As a result, the behaviors of these compounds are different each other, which are to be considered later.

Effect of Detergent Added to the Reaction System

It is known that a micelle stabilizes the open-lid conformation of a pancreatic lipase.[20] In order to make sure whether a detergent affects the enantioselectivity of a lipase, we investigated the effect of detergent on the lipase-catalyzed hydrolysis of racemic **1**. Triton X-100, a nonionic alkyl polyoxyethylene ether, was employed as a detergent. Since Triton X-100 has no charge, it may not interact electrostatically with amino acid residues of the lipase. The results are summarized in Table 3.

Table 3. Effect of Triton X-100 (TX100) on the Lipase-Catalyzed Hydrolysis.

$[E]_0$ (unit/ml)	$[S]_0$ (mmol/l)	TX100 (mM)	10^2k_R (h^{-1})	10^3k_S (h^{-1})	E-value
88.0	2.35	0.0	11.6 ±1.8	29.9 ±4.1	3.9 ±0.1
88.0	2.35	4.6	76.5 ±2.7	68.9 ±3.9	11.1 ±0.2
17.6	11.8	0.0	2.57 ±0.12	2.05 ±0.12	12.5 ±0.4
17.6	11.8	4.6	3.01 ±0.11	2.59 ±0.08	11.6 ±0.3
17.6	11.8	10.0	3.46 ±0.25	2.98 ±0.20	11.6 ±0.2

A detergent plays two roles in lipase-catalyzed hydrolysis: one on lipase, and the other on substrate. A detergent may stabilize the open-lid conformation by binding to the surface of a lipase.[20] The reaction system becomes a solution from a heterogeneous mixture by addition of the detergent. This observation demonstrates that the detergent binds to the substrate to make a complex. In the present study, the detergent enhanced both (*R*)- and (*S*)-activities. Furthermore, the detergent also enhanced the enantioselectivity under the conditions that $[S]_0/[E]_0$ is low (0.0267 μmol/unit), which reveals that the detergent certainly influences the structure of lipase. The effect of the detergent is related with the concentrations of lipase and substrate: under the conditions of high $[S]_0/[E]_0$ (0.670 μmol/unit), both (*R*)- and (*S*)-activities are enhanced by the detergent without the change in enantioselectivity. Thus, it seems that the detergent assists the lid of lipase to be opened by hydrophobic interaction, similarly to the hydrophobic substrate, **1**.

It is known that molecules of hexane and acetonitrile, non-substrate compounds, can bind to the surface or the active site of enzyme.[21,22] The hydrophobic addendum that enhances the rate of hydrolytic reaction, therefore, may interact to the lid of lipase and assist it to be opened, similarly to Triton X-100. The behavior of DMB has the largest and similar to that of the detergent. Cyclopentane, cyclohexane and diisopropyl ether are activators to an enantiomer, but inhibitors to the other one. Molecules of these addenda may interact to both the substrate-binding pocket and the lid. The lid locates close to the substrate-binding pocket of lipase and the

movement of the lid is related with the induced-fit.[23] Therefore, it is plausible that the molecules interacting to the lid, *that is*, existing at the substrate-binding pocket, cause the inhibition against the hydrolytic reaction of an enantiomer. Although we do not have data enough to discuss the behavior of cyclopentane and diisopropyl ether, the similarity in the effect of cyclopentane and diisopropyl ether on the lipase-catalyzed hydrolysis means that their interacting-sites on the lipase are close each other. Because both benzene and hexane inhibit the lipase-catalyzed hydrolysis, they may interact more prefentially to the substrate-binding pocket than DMB. Their efficiency of inhibition depends on the strength of hydrophobic interaction measured by their boiling points (hexane: 69 °C; benzene: 80 °C). The mechanism of enantioselective inhibition was postulated in the previous paper.[3]

Comparison of Enantioselectivities in Hydrolysis and Transesterification Catalyzed by a Lipase

It is often mentioned that enantioselectivities in (trans)esterifications catalyzed by a hydrolase in organic media are higher than those in the corresponding hydrolytic reactions in water.[24] In order to elucidate the correlation of mechanisms for transesterification and hydrolysis, we compared E-values of the transesterification between racemic sulcatol and vinyl acetate catalyzed by lipase PS in organic media[3] with those of the hydrolysis of racemic **1** catalyzed by lipase PPS in water. The correlation is shown in Fig. 4.

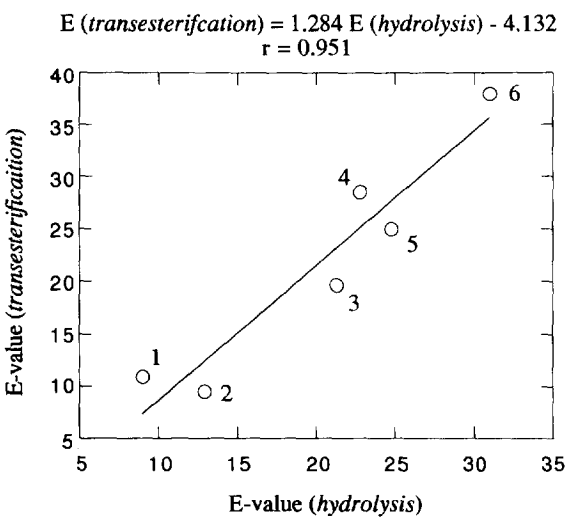


Figure 4. Correlation between E-values in the transesterifications and those in the hydrolyses catalyzed by lipase from *Pseudomonas cepacia*. Solvents (or addenda): (1) cyclohexane, (2) DMB, (3) diisopropyl ether, (4) hexane, (5) cyclopentane, and (6) benzene.

Two series of E-values that are estimated from different reactions are surprisingly correlated with each other, and the slope of the line is approximately 1. The meaning of slope = 1 is not so important, because

enantioselectivity of a lipase may be affected by its purity and immobilized state. On the other hand, high coefficient of correlation ($r = 0.951$) is worth notice. Thus, it is found that these addenda, in spite of their small quantities (192 ± 36 mmol/l), afford the same effect as that they do as a bulk solvent to the enantioselectivity of lipase. In other words, organic solvent influences the enantioselectivity as *a molecule* rather than as a bulk medium in the transesterification.[25]

In summary, we propose that a water-insoluble substrate (*e.g.* 2-acetoxy-6-methyl-5-heptene; **1**) improves the enantioselectivity of a lipase-catalyzed hydrolysis by stabilizing the open-lid conformation of the lipase. We have also shown that organic compounds that are used as reaction media behave as inhibitors or activators in the lipase-catalyzed hydrolysis. Thus, solvent dependence of the enantioselectivity stems from molecular interactions between particular sites of the lipase and molecules of the addendum or solvent. The site-specific interactions are more important than bulk interactions.

EXPERIMENTAL

Instruments

Gas chromatograms were recorded on a Shimadzu GC-14A Gas Chromatograph. For the analysis of enantiomeric excess in the substrate and product, a capillary column equipped with Chiraldex G-TA (Tokyo Kasei Kogyo Co., Ltd.) was employed.

Materials

Tris(hydroxymethyl)aminomethane, racemic 6-methyl-5-hepten-2-ol (sulcatol), Triton X-100 (Ave. M.W.: 602.5) and organic solvents were commercially available. Acetate of sulcatol (2-acetoxy-6-methyl-5-heptene) was obtained easily by chemical acetylation of sulcatol. Purified lipase PS (2,200,000 unit/g) was provided from Amano Pharmaceutical Co., Ltd.

Lipase PPS-Catalyzed Hydrolysis of 2-Acetoxy-6-methyl-5-heptene (**1**) *in Tris-HCl buffer ([E] = 17.6 unit/ml, [S] = 11.8 mmol/l)*

To obtain 88 unit/ml enzyme solution, lipase PPS (1 mg) was dissolved in 25 ml of Tris-HCl buffer (0.1 M, pH 8.0). The substrate (**1**, 10 mg) and Tris-HCl buffer (4 ml) were placed in a vial and, after addition of 1 ml of the enzyme solution to the vial, the resulting suspension was stirred magnetically at 30 °C. Five vials were provided for a kinetic measurement and an E-value. Periodically, the content of a vial was transferred into a separatory funnel, and the substrate and product were extracted with 20 ml of diethyl ether. After usual work-up, the remained mixture of **1** and sulcatol was analyzed with gas chromatography.

Kinetics

Enantiomeric excesses in **1** (ee_S) and the product (ee_P) were calculated from one chromatogram, since all

enantiomers were separated satisfactorily on this analysis. The rate constants (k_R and k_S) and E-values were calculated by the same mothod reported in the previous paper.[3]

ACKNOWLEDGMENT

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18. In this system, *ca.* 16 mg/ml of organic solvent is the limiting concentration to keep system in a dispersion state instead of a biphasic state. Because the biphasic state reduces the interaction between lipase and

hydrophobic compound, the concentration of organic solvent was kept at 16 mg/ml and the content in the vial was vigorously stirred in order to keep the dipersion state.

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