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# Studies on the Factors That Affect Stereoselective Esterification of (*R, S*) 2-Octanol with Octanoic Acid Catalyzed by Lipase in Organic Solvent

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## ABSTRACT

Stereoselective esterification of (*R, S*) 2-octanol with octanoic acid catalyzed by *Candida Sp* lipase (CSL) was carried out in cyclohexane. We have studied the effects of factors, such as temperature and the microenvironment of lipase, on this reaction. The results showed that CSL favored *R* enantiomer of (*R, S*) 2-octanol, and that the esterification activity and stereoselectivity of the lipase were dependent on these factors. The higher the temperature, the greater the esterification activity of CSL. A slight increase in stereoselectivity can be seen with temperature decrease. The optimal range of pH value for this reaction was 4.9–6.2. When the salt concentration was between 0 and 0.05 mol/L, CSL showed high activity. The salt concentration in the reaction system and the pH value at which CSL powder was prepared from the aqueous solution had no evident effect on the stereoselectivity of CSL.

The optimal range of the water content in the reaction system was 0.4–1.6%. The esterification activity and the stereoselectivity of CSL were enhanced 1.4-fold and 2.0-fold, respectively, by immediately removing the produced water. (*S*) 2-octanol with 95.2% enantiomeric excess (ee) was prepared. Based on these results, we have discussed why that all these factors affected this reaction.

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**Index Entries:** Lipase; esterification; stereoselectivity; organic media.

## INTRODUCTION

The most attractive characteristic that makes enzyme catalysts far superior to conventional chemical catalysts is their high stereoselectivity. The most frequent complaint about enzymes is that they must work in aqueous solutions. This is different from the everyday rules and customs of organic synthesis. For example, most compounds that organic chemists are interested in are insoluble in water. In addition, water often promotes unwanted side reactions and makes a number of common organic reagents decompose (*1*). With recent developments of enzyme technology, it has been observed that enzymes can work in organic solvents containing little or no added water (*2–5*). Because of this fact, enzymes can be applied successfully to organic synthesis. One of the major applications for enzyme catalysis in organic solvents is the resolution of racemic mixture. Stereoselective esterification catalyzed by enzymes has been developed in organic solvents (*6–9*). Klibanov (*1*) has reported the general mechanism of enzyme-catalyzed stereoselective esterification. By the use of this methodology, a lot of racemic fatty acids and alcohols have been resolved with enzymes catalysis in organic solvents (*6–12*). Bianchi et al. (*13*) have described the resolution of 2,3-epoxy alcohols using porcine pancreatic lipase in ethyl acetate or methyl propionate as the reaction medium. Epoxy alcohol was obtained with high enantiomeric excess. Porcine pancreatic lipase catalyzed the stereoselective esterification of 1.2- and 1.3-diols at the primary hydroxyl group in ethyl acetate, propionate, butyrate, or caprylate (*14*). Koshiro et al. (*8*) reported that the stereoselective esterification of (*R, S*) 2-(4-chlorophenoxy) propanoic acid was catalyzed by lipase OF 360. (*S*)-2-(4-chlorophenoxy) propanoic acid with 90% ee was obtained. It should be noted that the enantioselectivity of the enzyme can be controlled by the polarity of the solvent, the chain length of substrate, and the water content in the reaction system (15–17). How to regulate the enantioselectivity of enzyme is a key point in the preparation of chiral substances with high enantiomeric excess.

In this article, (*R, S*) 2-octanol and octanoic acid have been selected as the target substrates for lipase to catalyze the stereoselective esterification. This is important, because optically active 2-octanol is an excellent chiral building block. We have studied the effects of factors, such as the temperature and the enzyme microenvironment, on this stereoselective esterification catalyzed by *Candida Sp* lipase (CSL) in organic solvent. How these factors can affect the reaction has been explained based on the structure of the enzyme. This study is not only important in enhancing the stereoselectivity and the activity of lipase in organic solvent for industrial application, but also provides information for the study of enzyme structure and function.

## MATERIALS AND METHODS

### Materials

1. CSL was provided by the Institute of Microbiology, Academia Sinica, Beijing, China. The specific activity for CSL to hydrolyze olive oil is 5.25 U/mg solid.
2. Optically active 1-(1-naphthyl)ethyl isocyanate (NEI), (*R*)-NEI, and (*S*)-NEI were purchased from Aldrich Chemical Co., Ltd.
3. 4-Å molecular sieve was purchased from Shanghai UOP-UCC Molecular Sieve Company Limited, P. R. China.
4. PM30 film used for ultrafiltration was purchased from Pharmacia Company Limited, Sweden.
5. (*R, S*) 2-octanol and other reagents were all of analytical grade.

### Methods

*Determination of Hydrolysis Activity of Lipase*

The activity for lipase to hydrolyze olive oil was determined according to the method established by Kanasawud and Phutrakul (*18*). One unit of hydrolysis activity is defined as the amount of enzyme used to hydrolyze olive oil (0.23 g/mL) to liberate 1 μmol of fatty acid/min at 37°C.

*Stereoselective Esterification of (R, S) 2-Octanol with Octanoic Acid Catalyzed by CSL in Organic Solvent*

(*R, S*) 2-octanol (0.5 mL of 0.56 mol/L) and octanoic acid (0.5 mL of 0.8 mol/L) were mixed together in cyclohexane. Three milligrams of CSL were added into the mixture and incubated, with shaking (120 strokes/min), at 37°C for 4–5 d. The reaction was terminated by removing the enzyme.

*Determination of Esterification Activity of Lipase and Substrate Conversion* (%)

After the stereoselective of (*R, S*) 2-octanol with octanoic acid catalyzed by CSL was stopped by removing the enzyme, the substrate conversion (%) and the esterification activity of enzyme were calculated from the increase of the area of product peak, as determined by high-pressure liquid chromatography (HPLC) (Fig. 1). One unit of esterification activity was defined as the amount of enzyme that liberated $1 \times 10^{-9}$ mol product/min.

*Determination of Enantiomeric Excess (ee)*

The ee of 2-octanol was determined by normal-phase HPLC (*19*) using (*R*)-(−) or (*S*)-(+) NEI as chiral resolving reagent. 2-Octanol reacted with chiral NEI to give the corresponding diastereomeric carbamate that was separated on a μBorasil Silica column (id = 0.39 cm; length = 30 cm; eluent: 0.3% [v/v] ethanol/cyclohexane; flow rate: 1.0 mL/min.; detection wavelength: 254 nm). In the case of (*S*) NEI, (*R*) isomer of 2-octanol eluted more quickly than its (*S*) isomer, and vice versa for (*R*) NEI. The ee of the

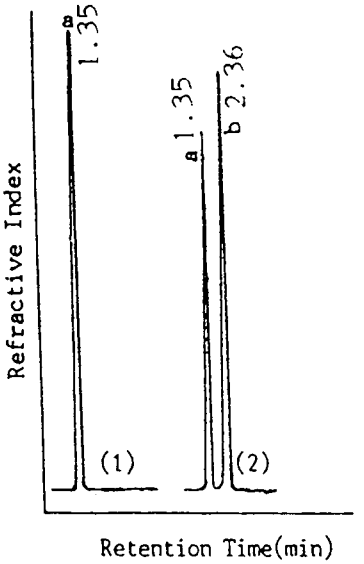



Fig. 1. Determination of esterification activity and substrate conversion (%) by HPLC. A mixture of (*R*, *S*) 2-octanol (0.5 mL of 0.56 mol/L) and octanoic acid (0.5 mL of 0.8 mol/L) was incubated at 37°C for a few days with CSL (3 mg) (*2*) or without CSL (*1*). The analysis was performed using $C_{18}$ column in 70% tetrahydrofuran, and the flow rate was 1.0 mL/min. Peak a: Octanoic acid and 2-octanol. Peak b: 2-octyl octanoate.

obtained esters was calculated from the ee of the unreacted 2-octanol according to the formula:

$$\%ee_{ester} = \%ee_{alcohol} / C - \%ee_{alcohol} \quad (1)$$

where C represents alcohol conversion (%).

### *Determination of the Enantiomeric Ratio, E Value*

The enantiomeric ratio, *E*, was calculated according to the formula (*20*):

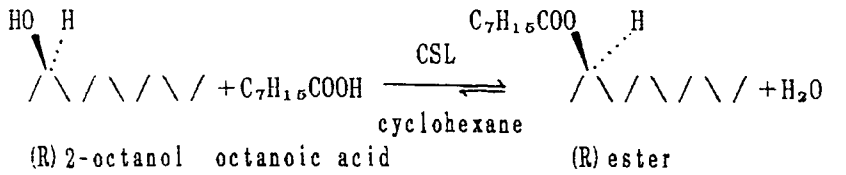
$$E = 1n[(1 - C)(1 - ee_S)] / 1n[(1 - C)(1 + ee_S)] \quad (2)$$

### *Removal of Water*

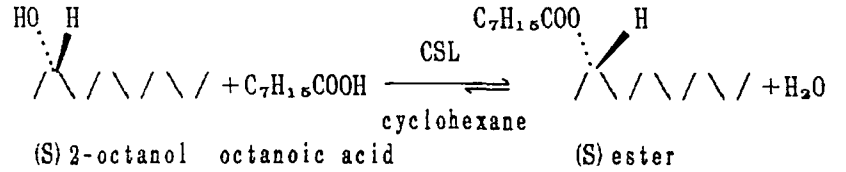
Removal of water from organic solvent and substrate was performed with 4 Å molecular sieves.

### *Preparation of CSL Powders from Enzyme Solutions with Different pH Values*

Crude CSL powders were dissolved at different pH values of 0.02 mol/L phosphate buffer and centrifuged at 6000 rpm for 15 min. The supernatants were ultrafiltrated with PM30 film in different pH values of 0.02 mol/L phosphate buffer, and then lyophilized into CSL powders.




Fast reaction




Slow reaction

Scheme 1

## RESULTS AND DISCUSSION

The processes that are involved in this kinetic resolution are depicted in Scheme 1. The fastest reacting enantiomer will be converted preferentially into the corresponding ester. If the stereoselectivity of lipase is high enough, the slow reaction will not take place. Under these conditions, (*S*) 2-octanol and (*R*) ester with high ee will be produced. How can we obtain enzyme with high enough stereoselectivity? It depends on the chain length of the substrate, the polarity of the organic solvent, particularly the microenvironment of the enzyme in the organic solvent, and the temperature. Some people have previously discussed the dependence of the stereoselective esterification catalyzed by enzymes in organic solvent on the substrate chain length and the polarity of organic solvent (*15–17*). In this article, we extended this work further to study the effects of other factors, such as the temperature and the microenvironment of the lipase in the organic solvent that was determined by the water content, the salt concentration in the reaction system, and the pH value at which the enzyme was prepared from aqueous solution, on the stereoselective esterification catalyzed by lipase in organic solvent.

### Effect of Temperature on the Stereoselective Esterification of (*R, S*) 2-Octanol Catalyzed by CSL in Organic Solvent

The stereoselective esterification of (*R, S*) 2-octanol (0.5 mL of 0.56 mol/L) with octanoic acid (0.5 mL of 0.8 mol/L) catalyzed by CSL (3 mg) was carried out at different temperatures according to Method 2. The results (Table 1) show that CSL favors *R* enantiomer of (*R, S*) 2-octanol.

Table 1
Effect of Temperature on the Stereoselective Esterification

| Temperature, °C | Reaction time, h | Alcohol conversion, % | Unreacted (*S*) alcohol ee, % | (*R*) ester ee, % | *E* | Relative esterification activity |
|---|---|---|---|---|---|---|
| 20 | 158 | 43.3 | 42.5 | 55.7 | 5.2 | 100.0 |
| 30 | 112 | 50.1 | 51.5 | 51.1 | 4.8 | 162.5 |
| 37 | 96 | 51.9 | 52.9 | 49.0 | 4.8 | 192.1 |
| 40 | 99 | 54.8 | 54.9 | 45.3 | 4.5 | 197.8 |
| 45 | 91 | 50.0 | 47.8 | 47.8 | 4.4 | 196.3 |
| 50 | 83 | 50.1 | 46.2 | 46.0 | 4.2 | 214.4 |
| 60 | 73 | 47.9 | 42.1 | 45.7 | 4.0 | 232.9 |

The water content in the reaction system was 0.4%.

(*S*) 2-octanol and *R* ester can be prepared. The esterification activity and the stereoselectivity of CSL are dependent on the temperature. The higher the temperature, the greater the esterification activity. A slight increase in stereoselectivity (*E* value) can be seen with temperature decrease. The results may be explained by three facts:

1. The denaturation process of enzymes requires water and therefore should not occur in a water-free environment. Dehydration makes lipase much more rigid and eliminates its conformational flexibility (*21,22*). Therefore, enzymes in organic solvents keep their appropriate folded conformations even at high temperature;
2. The stereoselective esterification of (*R, S*) 2-octanol catalyzed by CSL is kinetic. Temperature has an effect on the reaction rate. The temperature affects the fast reaction and the slow reaction (Scheme 1) differently;
3. Holmberg and Hult (*15*) have confirmed that the *E* value is an exponential function of the temperature, as can be seen from the equation

$$RT_1 \ln E_1 = RT_2 \ln E_2$$

Our experimental data are in agreement with this equation.

The effect of temperature on stereoselective esterification catalyzed by the enzyme can serve as a tool for enhancing ee in cases where a decrease in enzyme activity can be accepted.

## Effect of the Microenvironment of CSL on the Stereoselective Esterification

The microenvironment of enzyme is very important to enzyme-catalyzed reactions in organic solvent. An enzyme will not show activity and

Table 2
Effect of the Water Content in the Reaction System on the Stereoselective Esterification of (*R*, *S*) 2-Octanol with Octanoic Acid Catalyzed by CSL

| Water content, % | Reaction time, h | Alcohol conversion, % | Unreacted (*S*) alcohol ee, % | (*R*) ester ee, % | *E* | Relative esterification activity |
|---|---|---|---|---|---|---|
| 0.0 | 90 | 0.0 | — | — | — | 0.0 |
| 0.4 | 90 | 51.9 | 40.6 | 45.0 | 3.2 | 94.6 |
| 0.8 | 90 | 53.7 | 51.8 | 44.7 | 4.3 | 97.8 |
| 1.2 | 90 | 55.5 | 53.1 | 42.6 | 4.1 | 100.0 |
| 1.6 | 102 | 58.3 | 56.8 | 40.6 | 4.1 | 92.1 |
| 2.0 | 102 | 50.0 | 40.0 | 40.0 | 3.4 | 79.2 |
| 2.4 | 115 | 49.3 | 37.0 | 38.0 | 3.2 | 69.4 |
| 2.8 | 115 | 45.0 | 30.0 | 36.7 | 2.9 | 63.5 |

stereoselectivity, unless the active center in the microenvironment of enzyme is in the optimal ionization state. The ionization state of the active center is determined by the water content, the ionic strength in the reaction system, and the pH value at which the enzyme powder was prepared from the aqueous solution. Therefore, we have studied the dependence of the stereoselective esterification on the following three factors that affect the stereoselective esterification. We have discussed the effect of the microenvironment of the enzyme on this reaction.

### *Effect of the Water Content in the Reaction System on the Stereoselective Esterification*

CSL catalyzed the stereoselective esterification of (*R*, *S*) 2-octanol, and octanoic acid was carried out at different water content values, in the reaction system according to Method 2. It was observed (Table 2) that the water content in the reaction system can affect the esterification activity and the stereoselectivity (*E* value) of CSL. Without water in the reaction system, CSL does not show activity and stereoselectivity. The optimal range of the water content in the reaction system is between 0.4 and 1.6%. When the water content is above 1.6%, the higher the water content is, the poorer the activity and the stereoselectivity become. These results may be caused by (1) a thin layer of water in the microenvironment of enzyme, which is necessary to retain its catalytically active conformation in anhydrous media (22); with the water content in the microenvironment of enzyme increased, the unwanted side reaction, hydrolysis reaction, will be promoted, and (2) water affords a conformation flexibility to enzyme molecules. The high conformational flexibility of the enzyme, which is caused by excessive water in the microenvironment of the enzyme, shows such small steric constraints that the stereoselectivity of enzyme may be decreased to some extent.

Table 3
Effect of the Water in the Process of the Reaction on the Stereoselective Esterification

| Reaction time, | Alcohol conversion, % | | Unreacted (S) alcohol ee, % | | E | | Relative activity | |
|---|---|---|---|---|---|---|---|---|
| h | with MS | with GB | with MS | with GB | with MS | with GB | with MS | with GB |
| 0 | 0 | 48.1 | — | 45.8 | — | 4.5 | 0 | 70.5 |
| 20 | 68.2 | 55.1 | 95.2 | 54.8 | 8.8 | 4.4 | 100.0 | 80.8 |
| 40 | 63.3 | 53.2 | 87.2 | 52.1 | 8.2 | 4.3 | 92.8 | 78.0 |

All the reactions were carried out for 92 h.

The suggestions that too much water can promote the unwanted side reaction and that the conformational flexibility of enzyme afforded by water will decrease the stereoselectivity of the enzyme can be demonstrated experimentally. According to Method 2, we mixed 3 mg of CSL, 0.28 mol/L (*R*, *S*) 2-octanol, and 0.40 mol/L octanoic acid together in 1 mL cyclohexane containing 0.4% water. Before the reaction (0 h) and in the process of the reaction (after the reaction was carried out for 20 and 40 h), we added molecular sieves (MS) into the reaction mixture to remove the water. In the control experiment, we put the same number of glass beads (GB) into the reaction mixture. Data on this experiment are presented in Table 3. The results show that the esterification activity and the stereoselectivity (*E* value) of CSL can be enhanced 1.4- and 2.0-fold by immediately removing the produced water, and that the trace of water in the reaction system at the beginning of the reaction is necessary to promote the stereoselective esterification. (*S*) 2-octanol with 95.2% ee was prepared. This experimental result demonstrates the above suggestions may be correct.

### *Effect of the pH Value at Which CSL Powder Was Prepared from the Aqueous Solution on the Stereoselective Esterification*

The stereoselective esterification of (*R*, *S*) 2-octanol with octanoic acid catalyzed by CSL was carried out at 37°C by using a series of enzyme powders prepared according to Method 7. The results (Table 4) reveal that the pH value at which CSL powder is prepared from the aqueous solution has an effect on the CSL activity, but less of an effect on the stereoselectivity of CSL. The optimal range of pH values for this reaction is 4.9–6.2. These results may be explained by the facts that the pH state at which CSL powder was prepared from the aqueous solution can be retained in the microenvironment of the enzyme in the organic solvent and that the optimal pH of the enzyme microenvironment is necessary to retain ideal enzyme activity in the organic solvent. Therefore, the stereoselective esterification was dependent on the pH value at which the CSL powder was prepared. Moreover, compared to enzymes in aqueous solution, the rigid conformation of the enzyme in the organic solvent makes them obtuse to the pH value of the microenvironment of the enzyme. There is an optimal pH range for enzyme catalysis in organic solvents.

Table 4
Effect of pH on the Stereoselective Esterification

| pH | Reaction time, h | Alcohol conversion, % | Unreacted (*S*) alcohol ee, % | (*R*) ester ee, % | *E* | Relative esterification activity |
|---|---|---|---|---|---|---|
| 4.4 | 99.0 | 42.2 | 40.1 | 54.9 | 5.0 | 68.1 |
| 4.6 | 99.0 | 45.1 | 41.0 | 50.0 | 4.4 | 74.5 |
| 4.9 | 99.0 | 59.0 | 59.9 | 41.6 | 4.3 | 100.0 |
| 5.3 | 99.0 | 52.2 | 50.8 | 46.5 | 4.4 | 88.2 |
| 6.0 | 99.0 | 49.1 | 52.8 | 54.7 | 5.7 | 84.3 |
| 6.2 | 99.0 | 58.8 | 57.6 | 40.4 | 4.1 | 100.0 |
| 6.8 | 99.0 | 50.2 | 52.1 | 51.9 | 5.1 | 73.8 |
| 7.2 | 99.0 | 39.9 | 40.1 | 59.9 | 5.7 | 65.5 |

Table 5
Effect of the Salt Concentration on the Stereoselective Esterification

| Salt concentration, mol/L | Reaction time, h | Alcohol conversion, % | Unreacted (*S*) alcohol ee, % | (*R*) ester ee, % | *E* | Relative esterification activity |
|---|---|---|---|---|---|---|
| 0.00 | 108 | 59.8 | 65.0 | 43.7 | 4.8 | 100.0 |
| 0.01 | 108 | 57.8 | 59.2 | 43.2 | 4.5 | 97.8 |
| 0.05 | 108 | 56.1 | 54.0 | 42.3 | 4.1 | 91.9 |
| 0.13 | 108 | 51.2 | 50.6 | 48.2 | 4.6 | 84.0 |
| 0.25 | 108 | 48.2 | 41.6 | 43.9 | 3.9 | 71.2 |
| 0.50 | 108 | 48.2 | 45.2 | 48.8 | 4.4 | 67.5 |

### *Effect of the Salt Concentration on the Stereoselective Esterification*

Three milligrams of CSL, 0.28 mol/L (*R*, *S*) 2-octanol and 0.40 mol/L octanoic acid were mixed together in 1 mL of cyclohexane, then 4 μL distilled water containing different concentrations of sodium chloride were added into the mixture. The reaction was carried out according to Method 2. The results (Table 5) show that the salt concentration can affect the stereoselective esterification. When the salt concentration is between 0 and 0.05 mol/L, CSL shows similarly high activity. However, the activity of CSL decreases when the salt concentration is over 0.05 mol/L. Moreover, the salt concentration has no evident effect on the stereoselectivity of CSL. The results indicate that the salt concentration can determine the ion state of the enzyme microenvironment, on which the enzyme activity is dependent.

In conclusion, the stereoselective esterification of (*R*, *S*) 2-octanol with octanoic acid catalyzed by CSL was carried out in cyclohexane. We have studied the effect of the temperature and the microenvironment of

the lipase on the esterification activity and the stereoselectivity of CSL in cyclohexane. The results showed that the optimal range of the water content, the pH value, and the salt concentration were 0.4–1.6%, 4.9–6.2, and 0–0.05 mol/L, respectively.

The esterification activity and the stereoselectivity of CSL were enhanced 1.4- and 2.0-fold, respectively, by immediately removing the produced water. A slight increase in the stereoselectivity and a decrease in the esterification activity of CSL can be seen with temperature decrease. (*S*) 2-octanol with 95.2% ee was prepared. The reasons why all these factors can affect this reaction have been discussed.

## ACKNOWLEDGMENTS

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