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# Preparation of Interface-Assembled Carbonyl Reductase and Its Application in the Synthesis of S-Licarbazepine in Toluene/Tris-HCl Buffer Biphasic System

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In this study, interface-assembled carbonyl reductase (IACR) was prepared and used in the synthesis of S-licarbazepine in a toluene/Tris-HCl biphasic system. The carbonyl reductase (CR) was conjugated with polystyrene to form a surfactant-like structure at the interface of the toluene/Tris-HCl biphasic system. The interface-assembled efficiency of IACR reached 83% when the CR (180 U/mg) and polystyrene concentration were $8 \times 10^2$ g/ml and $3.75 \times 10^3$ g/ml, respectively. The conversion reached 95.6% and the enantiometric excess of S-licarbazepine was 98.6% when $3.97 \times 10^6$ nmol/l oxcarbazepine was converted by IACR using 6% ethanol as a co-substrate in toluene/Tris-HCl (12.5:10) at 30°C and 43 ×*g* for 6 h. IACR could be reused efficiently five times.

**Keywords:** Asymmetric reduction, interface-assembled carbonyl reductase, oxcarbazepine, polystyrene, S-licarbazepine

## Introduction

S-Licarbazepine acetate is approved as a voltage-gated sodium channel inhibitor for the treatment of epileptic seizures in adults [1]. S-Licarbazepine is a key intermediate of eslicarbazepine acetate and an active metabolite of oxcarbazepine [2]. The synthesis of S-licarbazepine has been studied using chemical and whole-cell biotransformation methods [3, 4]. Liu *et al*. [5] reported the synthesis of S-licarbazepine using expensive chiral Ru or Rh as a catalyst. Ravinder *et al*. [6] studied the synthesis of eslicarbazepine acetate using the R-2-Me-CBS catalyst. The preparation of a chemical chiral catalyst is complex and costly when using a precious metal as the raw material. Desai *et al*. [7] developed a process for the preparation of eslicarbazepine by resolution of racemic licarbazepine using acetyl mandelic acid. The highest achieved value of the conversion rate for the resolution of racemic licarbazepine is only 50%. Compared with the above method, whole-cell biotransformation in the asymmetric reduction of oxcarbazepine is considered a better method for the preparation of S-licarbazepine because the microorganism can be obtained easily and conversion can reach 100% theoretically [8, 9]. The whole-cell biotransformation of oxcarbazepine was carried out in an aqueous solution or an aqueous/organic solvent biphasic solution [4, 8]. We previously reported that S-licarbazepine was synthesized by the asymmetric reduction of oxcarbazepine with CGMCC No. 2266 in aqueous solution. The S-licarbazepine yield was $3.7678 \times 10^6$ nmol·l$^{-1}$·d$^{-1}$ in continuous reduction over 4 days. The enantiometric excess of S-licarbazepine was 100% [4]. Only a small amount of oxcarbazepine can be processed because it is difficult for oxcarbazepine to dissolve in aqueous solution. An aqueous/organic solvent biphasic solution was applied in whole-cell biotransformation to improve the productivity. Singh *et al*. [10] reported the asymmetric reduction of oxcarbazepine using whole-cell *Pichia methanolica* biotransformation in deionized water and hexane biphasic solution. The

conversion and enantiomeric excess of S-licarbazepine were both above 98% [10]. In this paper, interface-assembled carbonyl reductase (IACR) was used in the reduction of oxcarbazepine to prepare S-licarbazepine in an aqueous/organic solvent biphasic solution to improve the interfacial catalytic efficiency and reuse of the enzyme.

The catalytic ability of enzymes in membranes is the basis for the biological function of cells or organelles [11]. This phenomenon provides a good basis for the preparation of interface-assembled enzymes. By simulating the cell-membrane structure, enzymes can be attached to macromolecular polymers to prepare interface-assembled enzymes [12]. Interface-assembled enzymes contain both hydrophilic and hydrophobic groups, and are similar to surfactants with enzymes as the main body [13, 14]. Interface-assembled enzymes can be used as catalysts for biotransformation in an oil/water two-phase system, and accumulates at the oil/water interface, where the hydrophobic group of the macromolecular polymer extends to the oil phase and the enzyme embedded in the water phase [15, 16]. The interface-assembled enzyme plays a good catalytic function at the organic/water interface because a substrate that is dissolved in water or an organic phase can easily access the enzyme [5, 14]. The interface-assembled enzyme is the ideal biological catalyst in organic/water two-phase systems [14]. Wang *et al*. [12] reported that the interfacial assembly of alpha-chymotrypsin conjugated with polystyrene (PS), poly(methyl methacrylate), and poly(L-lactic acid) was examined using the pendant drop method. It was believed that the assembly involved two steps; the adsorption of the particulates and the subsequent rearrangement, dissociation, and redispersion of the conjugates at the interface. Zhu *et al*. [14] reported that native water-soluble enzymes were transformed into interface-binding enzymes via conjugation with hydrophobic polymers, thus enabling interesting interfacial biocatalysis between immiscible chemicals at oil/water interfaces. PS-conjugated beta-galactosidase showed a catalytic efficiency that was more than 145 times higher than that of the native enzyme for a transgalactosylation reaction [14]. In this paper, PS-conjugated carbonyl reductase was used in the asymmetric reduction of oxcarbazepine to prepare S-licarbazepine in an oil/water biphasic system.

S-Licarbazepine was synthesized using IACR as the catalyst. The mechanism of S-licarbazepine synthesis is shown in Fig. 1. S-Licarbazepine was synthesized by the asymmetric reduction of oxcarbazepine using IACR as a catalyst at the interface of the organic phase and aqueous phase. Cofactor NADH was regenerated using ethanol as a co-substrate. Ethanol was converted to acetaldehyde using ethanol dehydrogenase and an $NAD^+$-acquired hydride ion to form NADH. Oxcarbazepine can be converted efficiently with an adequate amount of NADH as a cofactor. The effects of the organic solvent, carbonyl reductase (CR) concentration, and PS concentration on the interface-assembled efficiency of IACR were studied. Then, the biotransformation process for S-licarbazepine synthesis was optimized.

## Materials and Methods

### Reagents and Materials

Oxcarbazepine and S-licarbazepine were purchased from Shanghai ZiQi Biological Technology Co. (China) PS and ethanol dehydrogenase (200 units/mg) were purchased from Aladdin Industrial Corporation (China). *Bacillus anthracis* CGMCC No. 12337 was screened from soil at the Zhejiang University of Technology, and was preserved at the China General Microbiological Culture Collection Center (CGMCC).

### Preparation of free Carbonyl Reductase

The solid medium for microbial preservation was composed of $3 \times 10^3$ g/ml yeast extract, $5 \times 10^3$ g/ml $(NH_4)_2SO_4$, $2.5 \times 10^2$ g/ml $MgSO_4$, $1 \times 10^3$ g/ml $K_2HPO_4 \cdot 3H_2O$, $1 \times 10^3$ g/ml $KH_2PO_4$, and $2.0 \times 10^4$ g/ml agar. The liquid solution for the microbial culture was composed of $3.0 \times 10^4$ g/ml glucose, $1.5 \times 10^4$ g/ml peptone, $2.5 \times 10^2$ g/ml $MgSO_4$, $1 \times 10^3$ g/ml $K_2HPO_4 \cdot 3H_2O$, and $1 \times 10^3$ g/ml $KH_2PO_4$. *Bacillus anthracis* CGMCC No. 12337 picked from the solid medium was inoculated into 100 ml of liquid solution and cultivated in a 30°C–97×*g* shaker for 24 h. The cells obtained above were transferred into 500 ml of fermentation solution with a 10% inoculum concentration. After being cultivated for 36 h, the cells were harvested using centrifugation (8,000 ×*g*, 10 min).

Cell sediment (dry weight = 5 g) dispersed in 100 ml of Tris-HCl buffer (0.02 mol/l, pH 7.0) was processed by an ultrasonic processor to break the cells. Then, the disrupted solution was centrifuged (8,000 ×*g*, 10 min). The supernatant containing CR was processed by slow stirring in an ice-water bath for 2 h. The CR was precipitated when the ammonium sulfate saturation was 60%, and it was dissolved in 2 ml of Tris-HCl buffer (0.02 mol/l, pH 7.0). The specific activity of CR was 180 units/mg, and was determined using the methods described below.

### Preparation of Interface-Assembled Carbonyl Reductase [14]

A 5-ml Tris-HCl buffer (0.02 mol/l, pH 7.0) containing 1 ml of 180 units/mg CR was added to 5 ml of an organic solvent containing 1–8 mg PS (PS, Mw 10,000 Da). The reaction was carried out at 43 ×*g* and 30°C for 1 h in the dark. The CR-PS was assembled at the interfacial region after centrifugation (10,000 ×*g*, 10 min). The interfacial area was 16.6 $cm^2$. The interfacial region was further purified by washing three times using a Tris-HCl buffer and an organic solvent to remove residual free CR and PS. IACR was used as the catalyst during the biotransformation of oxcarbazepine.

**Assay of Protein Content and Activity of CR**

The protein content of the enzyme was determined using the Bradford method with bovine serum albumin as the standard [17]. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the conversion of 1 μmol NADH to $NAD^+$ per minute at 25°C. Then, 100 μl of enzymes was added to 5 ml of 0.02 mol/l Tris-HCl buffer (pH 7.9) containing $2.5 \times 10^5$ nmol/l NADH and $2.5 \times 10^5$ nmol/l acetophenone at 25°C for 5 min. The variation in the absorbance over 1 min at 340 nm was determined using a microplate reader. The specific enzyme activity can be calculated using Eqs. (1) and (2).

$$\text{Activity (U)} = E \times V \times 10^3/(6{,}220 \times 1) \quad (1)$$

$$\text{Specific activity of enzyme (U/mg)} = \text{Activity/protein content} \quad (2)$$

where $E$ represents the absorbance change over 1 min at 340 nm, $V$ is the volume of the reaction mixture, the molar absorption coefficient is 6,220 $l \cdot mol^{-1} \cdot cm^{-1}$, and the optical distance is 1 cm.

**Assay of Protein Content and Activity of IACR**

The protein content of IACR can be calculated using the material balance. The protein content of the initial CR and the residual free CR in the Tris-HCl buffer were detected using the Bradford method. The IACR obtained using the method described above was added to 5 ml of 0.02 mol/l Tris-HCl buffer (pH 7.9) containing $2.5 \times 10^5$ nmol/l NADH and $2.5 \times 10^5$ nmol/l acetophenone at 25°C for 5 min. The absorbance change over 1 min at 340 nm was determined using a microplate reader. The specific enzyme activity was calculated using Eqs. (1) and (2).

**Asymmetric Reduction of Oxcarbazepine in Preparation of S-Licarbazepine**

Next, $1.98\sim9.52 \times 10^6$ nmol/l oxcarbazepine was added to a 100-ml shaking flask containing 10 ml of Tris-HCl buffer (0.02 mol/l, pH 3.0–8.0), 3–20 ml of organic solvent, 0.02–0.09 mol/l NADH, 0.2 mg of ethanol dehydrogenase (200 U/mg), and 0–7% co-substrates. The reaction was carried out at 30°C and 43 ×*g* for 3–10 h. The Tris-HCl buffer phase and organic phase were layered clearly after centrifugation (10,000 ×*g*, 10 min). After filtration, the organic phase containing S-licarbazepine was analyzed using HPLC. Ethyl acetate was used three times to extract S-licarbazepine from the Tris-HCl buffer phase. Oxcarbazepine is insoluble in water, and it cannot be detected in ethyl acetate. However, it can be analyzed in toluene by HPLC. S-Licarbazepine was distributed not only in toluene but also in Tris-HCl buffer, and the S-licarbazepine content in both the Tris-HCl buffer and toluene was detected. Finally, the extract and organic phase were mixed, and the organic solvent was distilled. S-Licarbazepine was concentrated and analyzed using HPLC. The contents of S-licarbazepine and oxcarbazepine were then used to calculate the conversion.

**Analysis Methods**

The conversion and enantiometric excess of S-licarbazepine were analyzed using HPLC equipped with a chiral OD-H column (4.6 mm × 25 cm × 5 μm; Daicel, Japan). The mobile phase was hexane containing 0.05% THF/isopropanol (98:2). The detection wavelength was 210 nm. The flow rate of the mobile phase was 0.8 ml/min. The column temperature was 25°C. The injection volume of the sample was 10 μl.

**Comparison Experiment with Free CR or IACR as Catalysts in Aqueous or Tris-HCl/ Toluene Biphasic System**

IACR was prepared as described above. The conditions for its preparation were as follows: $3.75 \times 10^3$ g/ml PS was added to 10 ml of toluene to form the organic phase. Then, $8 \times 10^2$ g/ml CR (180 U/mg) was dissolved in 10 ml of Tris-HCl buffer (0.02 mol/l, pH 7.0) to form an aqueous phase. The area of the interface was 16.6 $cm^2$. The specific activity of IACR was 150 U/mg. To compare with IACR, $8 \times 10^2$ g/ml CR (180 U/mg) was suspended in the biphasic or aqueous system to convert oxcarbazepine.

The biphasic system reaction conditions were as follows: 0.08 mol/l NADH, 6% ethanol, and 0.2 mg of ethanol dehydrogenase (200 U/mg) were dissolved in 10 ml of the aqueous phase obtained above. Then, $3.97 \times 10^6$ nmol/l oxcarbazepine was dissolved in 12.5 ml of the organic phase obtained above. IACR (150 U/mg, 16.6 $cm^2$) and $8 \times 10^2$ g/ml CR (180 U/mg) were used as the catalysts in the Tris-HCl/ toluene biphasic system. The reaction was carried out at 30°C and 43 ×*g*.

The aqueous system reaction conditions were as follows: $3.97 \times 10^6$ nmol/l oxcarbazepine, 0.08 mol/l NADH, 6% ethanol, 0.2 mg of ethanol dehydrogenase (200 U/mg), and $8 \times 10^2$ g/ml CR (180 U/mg) were added to 10 ml of Tris-HCl at 30°C and 43 ×*g*. IACR (150 U/mg, 16.6 $cm^2$) and $8 \times 10^2$ g/ml CR (180 U/mg) were used as catalysts in the aqueous system.

## Results

**Optimization of the Preparation Process of IACR**

The process for the preparation of IACR was optimized. The effects of the organic solvent, CR concentration, and PS concentration on the interface-assembled efficiency of IACR were studied in detail.

**Effect of organic solvent on the interface-assembled efficiency of IACR.** The interface-assembled efficiency of IACR was affected by the organic solvent. The solubility values of PS in various organic solvents were different. The low solubility values of PS in hexane, heptane, butanol, and dibutyl phthalate led to low specific activities of IACR. PS easily dissolves in benzene, toluene, dichloromethane, and chloroform, so the enzyme can be connected to the PS easily at the interface of the Tris-HCl and organic solvent. The specific activities of IACR were higher with toluene, dichloromethane, and chloroform as the organic solvent. The interface-assembled efficiency was defined as the ratio of the specific activity of IACR to the specific activity of CR.

**Table 1.** Effects of organic solvents on the specific activity of the interface-assembled carbonyl reductase (IACR) and interface-assembled efficiency.

| Organic solvent/Tris-HCl | Specific activity of IACR (U/mg) | Interface assembled efficiency of [a]IACR (%) |
|---|---|---|
| Toluene/Tris-HCl | 150 | 83 |
| Benzene/Tris-HCl | 72 | 40 |
| Dichloromethane/Tris-HCl | 129 | 72 |
| Chloroform/Tris-HCl | 119 | 66 |
| Hexane/Tris-HCl | 18 | 10 |
| Dibutyl phthalate/Tris-HCl | 14 | 8 |
| *n*-Butanol/Tris-HCl | 14 | 8 |
| *n*-Heptane/Tris-HCl | 16 | 9 |

[a]Conditions for IACR preparation: $8 \times 10^2$ g/ml free CR (180 U/mg), $3.75 \times 10^3$ g/ml PS, 16.6 $cm^2$, 80 rpm, 30°C, volume ratio of organic solvent and Tris-HCl buffer is 1:1.

The specific activity of free CR was 180 U/mg. Table 1 shows that the interface-assembled efficiency reached 83% and the specific activity of IACR was 150 U/mg with toluene as the organic solvent. Toluene is the optimum organic solvent for the preparation of IACR. IACR may show a different steric conformation at the interface of Tris-HCl and different organic solvents. At the interface of Tris-HCl and toluene, IACR exhibited a high specific activity. The reason for the effect of organic solvent on the interface-assembled efficiency of IACR is complex, and may be related to the polarity and toxicity of the organic solvent.

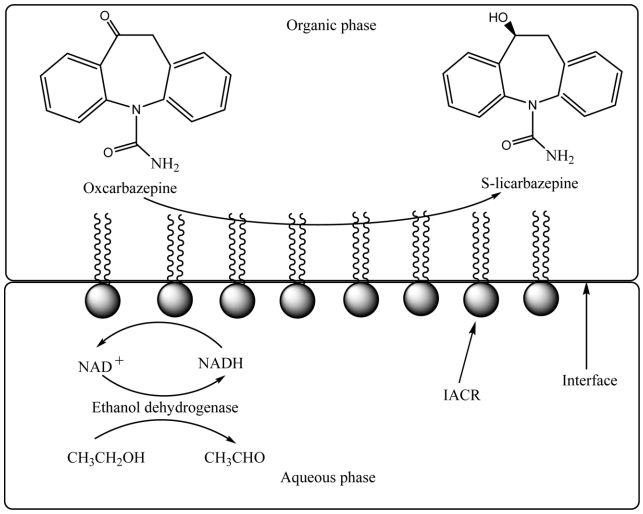


**Fig. 1.** Mechanism of S-licarbazepine synthesis by asymmetric reduction of oxcarbazepine with IACR as catalyst.

Toluene/Tris-HCl was selected as the optimal reaction biphasic system for further study.

**Effect of initial concentration of free CR on the protein self-assembly ratio and interface-assembled efficiency of IACR.** During the process of IACR preparation, the initial concentration of free CR in aqueous phase significantly affects the interface-assembled efficiency of IACR. The optimal initial concentration of free CR will benefit the connection of CR with PS at the interface region of Tris-HCl and toluene. The protein self-assembly ratio is defined as the ratio of the protein content of IACR and free CR in the Tris-HCl buffer. A high protein self-assembly ratio indicates that a larger amount of CR is connected to the PS. The protein self-assembly ratio decreased with an increase in the initial concentration of free CR (Fig. 2). The interface-assembled efficiencies of IACR were about 85% at different initial protein concentrations of free CR. The optimal initial protein concentration of free CR was $8 \times 10^2$ g/ml.

**Effect of PS concentration on the protein self-assembly ratio and interface-assembled efficiency of IACR.** Polymer-enzyme conjugates can self-assemble at oil/water interfaces and effect interfacial biotransformation. A surfactant-like structure consisting of a hydrophilic protein head and a hydrophobic polymeric tail is desirable for the construction of the interfacial-assembled enzyme. It was reported that the types and molecular weights of polymers significantly affect the specific activity of the interfacial-assembled enzyme [10, 22]. The optimal PS concentration is advantageous

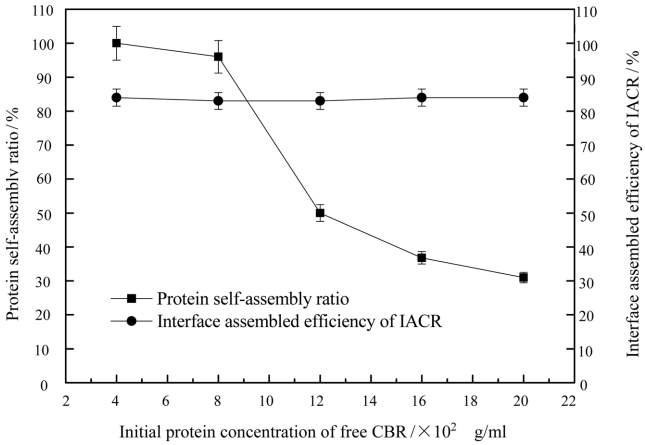


**Fig. 2.** Effect of initial concentration of free CR on the protein self-assembly ratio and the interface assembled efficiency of IACR.

Conditions for IACR preparation: free CR 180 U/mg, $3.75 \times 10^3$ g/ml PS, 16.6 $cm^2$, 43 ×*g*, 30°C, volume ratio of toluene and Tris-HCl buffer is 1:1.

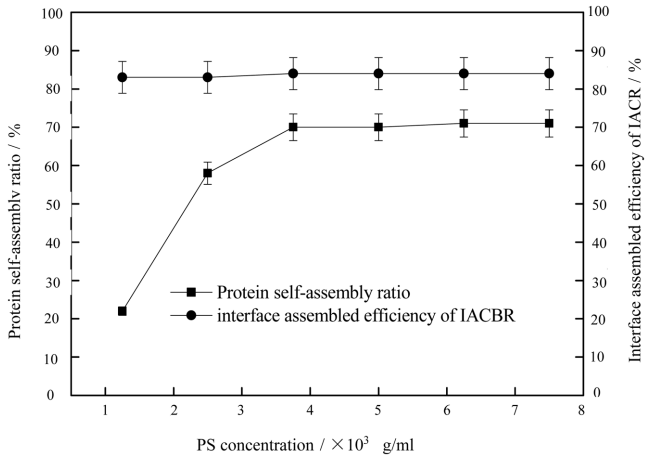


**Fig. 3.** Effect of PS concentration on the protein self-assembly ratio and the interface assembled efficiency of IACR.

Conditions for IACR preparation: $8 \times 10^2$ g/ml free CR (180 U/mg), 16.6 cm$^2$, 43 ×*g*, 30°C, volume ratio of toluene and Tris-HCl buffer is 1:1.

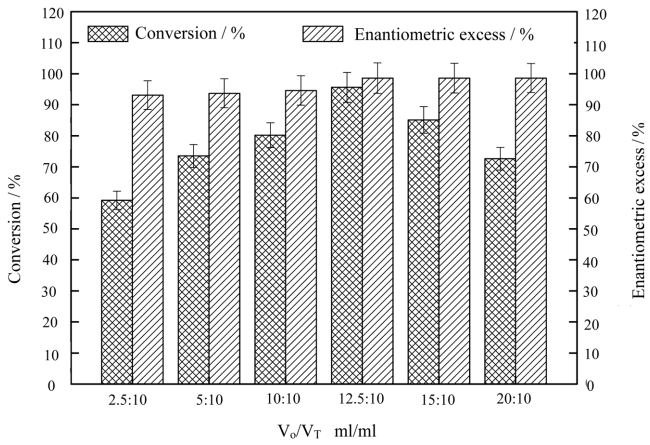


**Fig. 4.** Effect of the volume ratio of toluene and Tris-HCl buffer on reduction.

Reaction condition: Specific activity of IACR 150 U/mg, 16.6 cm$^2$, $3.97 \times 10^6$ nmol/l oxcarbazepine, 0.08 mol/l NADH, 6% ethanol, 0.2 mg ethanol dehydrogenase, 43 ×*g*, 6 h, 30°C.

during the preparation of IACR as free CR cannot be conjugated sufficiently when the PS concentration is low. The protein self-assembly ratio increased with an increase of the PS concentration (Fig. 3). Excessive PS unconjugated with CR will be removed. The optimal PS concentration was $3.75 \times 10^3$ g/ml. IACR was prepared when the PS concentration and initial concentrations of free CR were $3.75 \times 10^3$ g/ml and $8 \times 10^2$ g/ml, respectively. The IACR that was obtained was used in the asymmetric reduction of oxcarbazepine to synthesize S-licarbazepine.

## Optimization of the Reduction Process of Oxcarbazepine with IACR

**Effect of the volume ratio of toluene and Tris-HCl buffer on the reduction of oxcarbazepine.** The value of $V_o/V_T$ was defined as the volume ratio of toluene and the Tris-HCl buffer, where $V_o$ and $V_T$ are the volume of toluene and the Tris-HCl buffer, respectively. The value of $V_o/V_T$ affects the biological catalytic efficiency [18].

Fig. 4 shows that the conversion and enantiometric excess of S-licarbazepine increased as $V_o/V_T$ increased when the value of $V_o/V_T$ was lower than 12.5:10. The value of $V_o/V_T$ significantly affected the reduction conversion and the configuration of S-licarbazepine. The conversion and enantiometric excess of S-licarbazepine both achieved a maximum of 95.6% and 97.9% when the value of $V_o/V_T$ was 12.5:10. Therefore, the optimal volume ratio of toluene and Tris-HCl buffer was 12.5:10.

The content of oxcarbazepine in the Tris-HCl buffer was zero in the toluene/Tris-HCl buffer system. However, S-licarbazepine was detected in Tris-HCl buffer. Fig. 5 shows the concentration of S-licarbazepine in the Tris-HCl buffer for different values of $V_o/V_T$ after reduction. The concentration of S-licarbazepine in the Tris-HCl buffer decreased with increasing $V_o/V_T$ value. After reduction, the concentrations of S-licarbazepine distributed in the toluene and the Tris-HCl buffer were $3.87 \times 10^6$ and

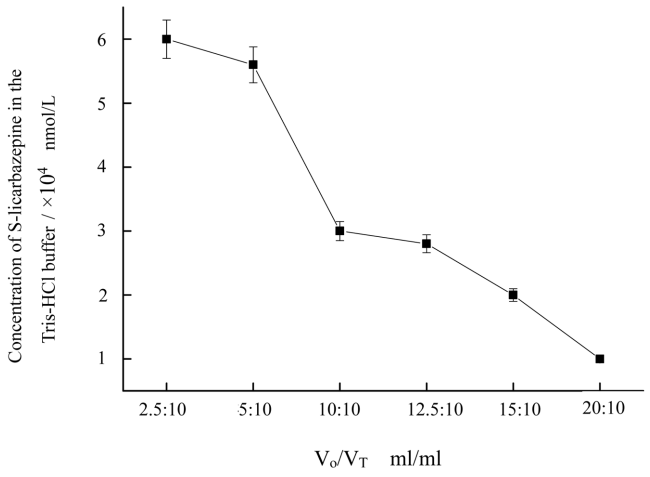


**Fig. 5.** Concentration of S-licarbazepine in the Tris-HCl buffer at the different value of $V_o/V_T$.

Reaction condition: Specific activity of IACR 150 U/mg, 16.6 cm$^2$, $3.97 \times 10^6$ nmol/l oxcarbazepine, 0.08 mol/l NADH, 6% ethanol, 0.2 mg ethanol dehydrogenase, 80 rpm, 6 h, 30°C.

$2.80 \times 10^4$ nmol/l, respectively, when the volume ratio of toluene and Tris-HCl buffer was 12.5:10. The concentration of S-licarbazepine in the toluene was much higher than that in the Tris-HCl buffer. Therefore, the concentration of S-licarbazepine was determined by the mixture of the extract and organic phase to calculate the conversion later in the study.

**Effect of the NADH concentration on reduction.** NADH was added to the Tris-HCl buffer as a cofactor to act as a reducer in the reduction of oxcarbazepine [19]. A different concentration of NADH was added to the Tris-HCl buffer to determine the effect of NADH on reduction. Oxcarbazepine could be converted to S-licarbazepine without the addition of NADH, which illustrates that the cofactor NADH was present in IACR. The addition of larger quantities of NADH improved the conversion. Fig. 6 shows that the conversion increased gradually with the increase of NADH concentration, and NADH provided hydrogen for reduction. First, 6% ethanol and 0.2 mg ethanol dehydrogenase were added to the Tris-buffer for the regeneration of NADH. Next, 0.08 mol/l NADH was found to be the optimal concentration, and was sufficient for reduction. The NADH concentration had no effect on the enantiometric excess of S-licarbazepine, which was maintained at about 97.9% for different NADH concentrations.

**Effect of co-substrate on reduction.** The use of a co-substrate is advantageous for the regeneration of NADH [20]. Glucose, sucrose, ethanol, methanol, and isopropanol were added to the Tris-HCl buffer as a co-substrate during the reduction process. The conversion reached 95.6% with the addition of 6% ethanol (Fig. 7). The optimal co-substrate is beneficial for the regeneration of the cofactor NADH. The cofactor regeneration mechanism is shown in Fig. 1. Ethanol dissolve easily in the water phase in a toluene/Tris-HCl biphase system because ethanol is a polar molecule. Most of the ethanol existing in the water phase is therefore beneficial for cofactor regeneration. Ethanol was converted to acetaldehyde using ethanol dehydrogenase, and cofactor $NAD^+$ was transformed to NADH. NADH was regenerated to provide hydrogen for the reduction of oxcarbazepine. Different co-substrates had almost no influence on the enantiometric excess of S-licarbazepine (Fig. 7). Therefore, the optimum co-substrate was 6% ethanol.

**Effects of substrate concentration and reaction time on reduction.** The substrate concentration significantly affects the reduction. A high concentration of substrate is toxic to the interface-assembled enzyme, whereas a low concentration of substrate leads to low productivity [21]. The effect of different substrate concentrations on the reduction was investigated using the toluene/Tris-HCl biphasic system. The conversion exceeded 90% after 6 h when the oxcarbazepine concentration was increased from $1.98 \times 10^6$ to $3.97 \times 10^6$ nmol/l (Fig. 8). Then, the conversion

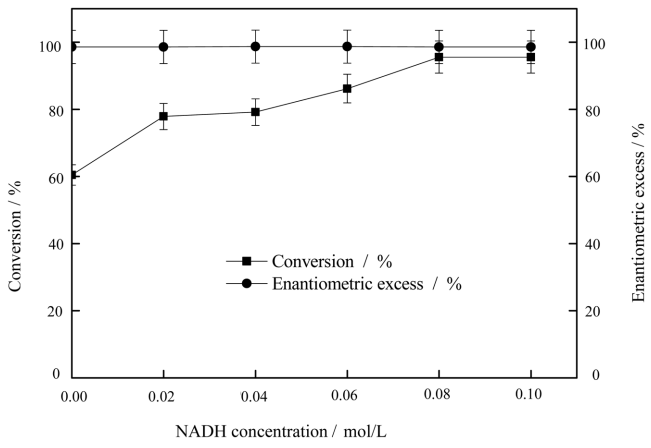


**Fig. 6.** Effect of NADH on reduction.

Reaction condition: Specific activity of IACR 150 U/mg, 16.6 $cm^2$, $3.97 \times 10^6$ nmol/l oxcarbazepine, 6% ethanol, 0.2 mg ethanol dehydrogenase, volume of biphase system 22.5 ml ($V_o/V_T$ 12.5:10), 43 ×*g*, 6 h, 30°C.

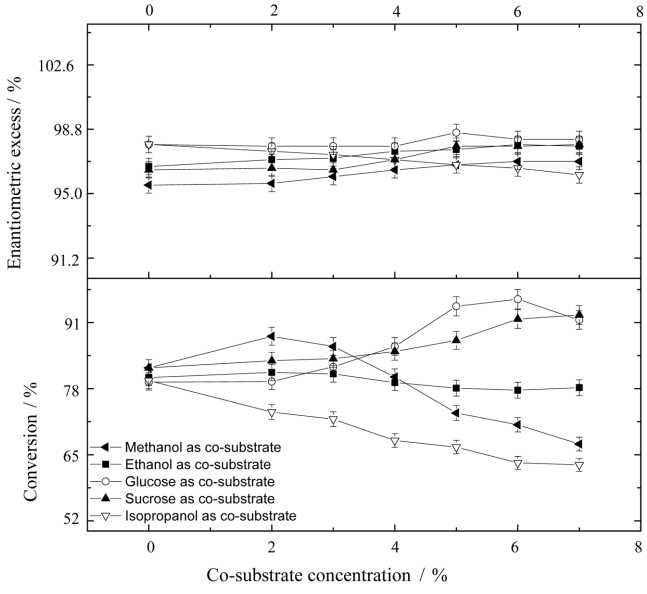


**Fig. 7.** Effect of different co-substrate on conversion and enantiometric excess of S-licarbazepine.

Reaction condition: Specific activity of IACR 150 U/mg, 16.6 $cm^2$, $3.97 \times 10^6$ nmol/l oxcarbazepine, 0.08 mol/l NADH, 0.2 mg ethanol dehydrogenase, volume of biphase system 22.5 ml ($V_o/V_T$ 12.5:10), 80 rpm, 6 h, 30°C.

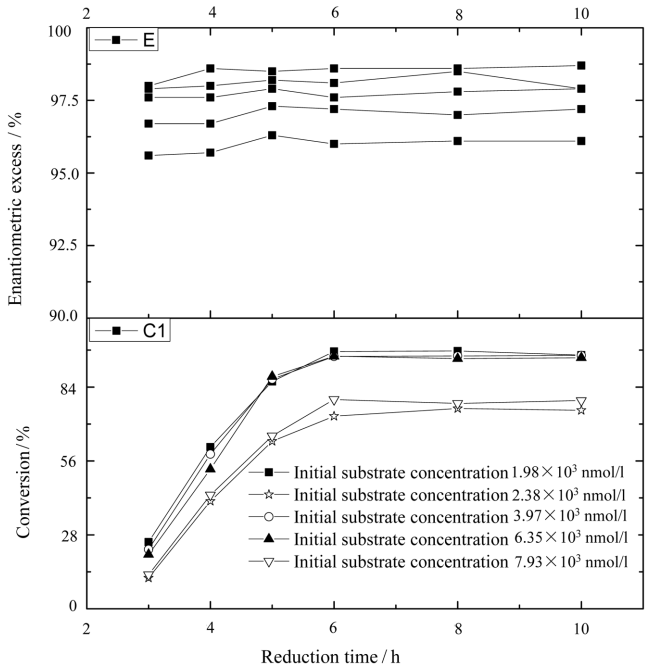


**Fig. 8.** Effect of substrate concentration on conversion and enantiometric excess of S-licarbazepine.

Reaction condition: Specific activity of IACR 150 U/mg, 16.6 $cm^2$, 0.08 mol/l NADH, 6% ethanol, 0.2 mg ethanol dehydrogenase, volume of biphase system 22.5 ml ($V_o/V_T$ 12.5:10), 80 rpm, 30°C.

decreased significantly when the oxcarbazepine exceeded $3.97 \times 10^6$ nmol/l. The enantiometric excess of S-licarbazepine was within the range of 95.6%–98.2% when the substrate concentration was $1.98 \times 10^6$~$3.97 \times 10^6$ nmol/l (Fig. 8). Therefore, the optimum substrate concentration was $3.97 \times 10^6$ nmol/l. The optimal reaction time was 6 h.

**Effect of temperature on reduction.** The temperature can affect the activity and stability of the enzyme as well as the reaction equilibrium [22, 23]. The conversion and enantiometric excess of S-licarbazepine decreased when the temperature was over 30°C. The interface-assembled enzyme may have lost its activity at high temperature. The optimal temperature was 30°C.

**Reuse of IACR in the toluene/Tris-HCl biphasic system.** Next, $3.97 \times 10^6$ nmol/l oxcarbazepine was converted using IACR in 22.5 ml of toluene/Tris-HCl ($V_o/V_T$ 12.5:10) containing 0.08 mol/l NADH, 6% ethanol, and 0.2 mg ethanol dehydrogenase at 43 ×*g* for 10 h. Toluene and Tris-HCl were removed. IACR was obtained by washing three times with Tris-HCl buffer and organic solvent alternately. IACR was redistributed in 22.5 ml of toluene/Tris-HCl ($V_o/V_T$ 12.5:10) containing $3.97 \times 10^6$ nmol/l oxcarbazepine, 0.08-mol/l NADH, 6% ethanol, and 0.2 mg of ethanol

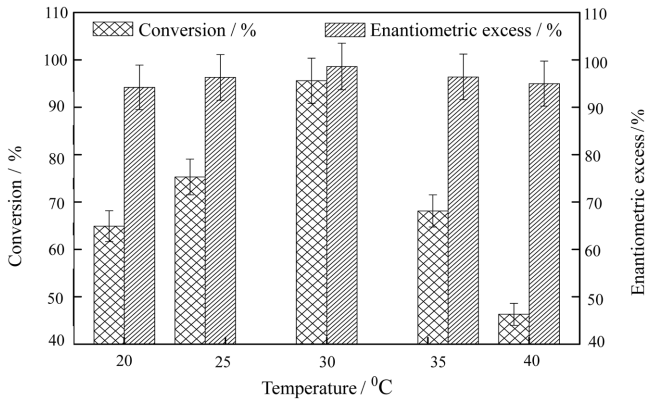


**Fig. 9.** Effect of temperature on reduction.

Reaction condition: Specific activity of IACR 150 U/mg, 16.6 $cm^2$, 0.08 mol/l NADH, $3.97 \times 10^6$ nmol/l oxcarbazepine, 6% ethanol, 0.2 mg ethanol dehydrogenase, volume of biphase system 22.5 ml ($V_o/V_T$ 12.5:10), 43 ×*g*, 6 h.

dehydrogenase. The above process was repeated every 6 h, and the conversion and enantiometric excess of S-licarbazepine was determined every time. The conversions were above 80% when IACR was used five times, while the enantiometric excess of S-licarbazepine was above 98%. IACR can be reused efficiently five times.

**Comparison of biotransformation processes in aqueous and in toluene/Tris-HCl biphasic systems using free CR and IACR as catalysts.** The biotransformation of oxcarbazepine was studied using free CR and IACR in aqueous and toluene/Tris-HCl biphasic systems in order to determine the catalytic efficiency of IACR. The conversion obtained with IACR as the catalyst in the toluene/Tris-HCl biphasic system was high. The conversion decreased rapidly after 5 h with free CR as the catalyst in the toluene/Tris-HCl biphasic system. It is believed that the free enzyme lost the activity in the presence of toluene after 5 h. The enantiometric excess of S-licarbazepine was above 95% with free CR or IACR in the aqueous or in the toluene/Tris-HCl biphasic system. This indicates that IACR was suitable for the synthesis of S-licarbazepine in the toluene/Tris-HCl biphasic system.

## Discussion

Free CR (180 U/mg) was isolated from *Bacillus anthracis* CGMCC No.12337 using cell disruption and ammonium sulfate precipitation, and then conjugated using PS at the interface of toluene and Tris-HCl to prepare the IACR. The conditions of IACR preparation, including the organic solvent, CR concentration, and PS concentration, were

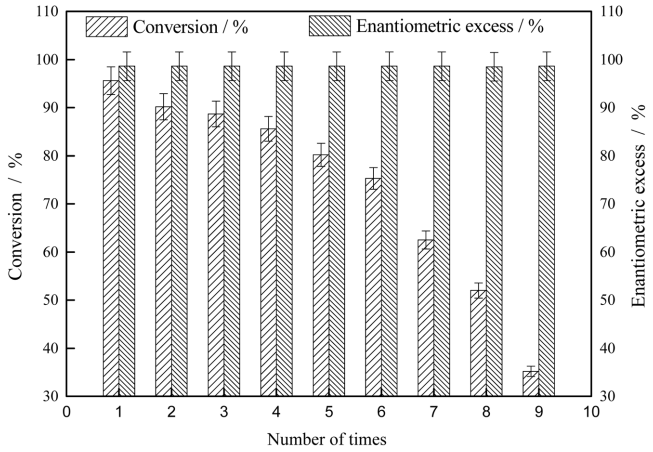


**Fig. 10.** Reuse of IACR in asymmetric reduction of oxcarbazepine in toluene/Tris-HCl.

optimized. An enzyme in water retains its original conformation, thus maximizing its enzyme activity. However, the conformation of an enzyme will change in the presence of an organic solvent, which decreases the enzyme activity. Moreover, the organic solvent removes essential water on the surface of the enzyme, which further decreases the enzyme activity. The loss of the enzyme activity was not high at the optimal organic solvent. Compared with benzene, dichloromethane, chloroform, hexane, dibutyl phthalate, *n*-butanol, and *n*-heptane, toluene showed good potential for the preparation of IACR. Toluene, dichloromethane, and chloroform are good organic solvents for dissolving PS, and $3.75 \times 10^3$ g/ml of PS can dissolve in toluene completely. PS dissolves easily in toluene and so a sufficient number of molecules of PS are arranged on the interface, which improves the interface-assembled efficiency. A monomolecular layer of IACR was formed by the conjunction of the hydrophobic part of the enzyme with the PS molecule at the interface of the oilwater phase. The natural conformation of the enzyme changed, and thus the activity of the enzyme decreased. The toluene/Tris-HCl was selected as an optimal reaction biphasic system for IACR preparation. The full contact of molecules of PS and CR is good for the formation of IACR. The optimal CR and PS concentrations were $8 \times 10^2$ g/ml and $3.75 \times 10^3$ g/ml. The specific activity of IACR and the interface-assembled efficiency reached 150 U/mg and 83%, respectively.

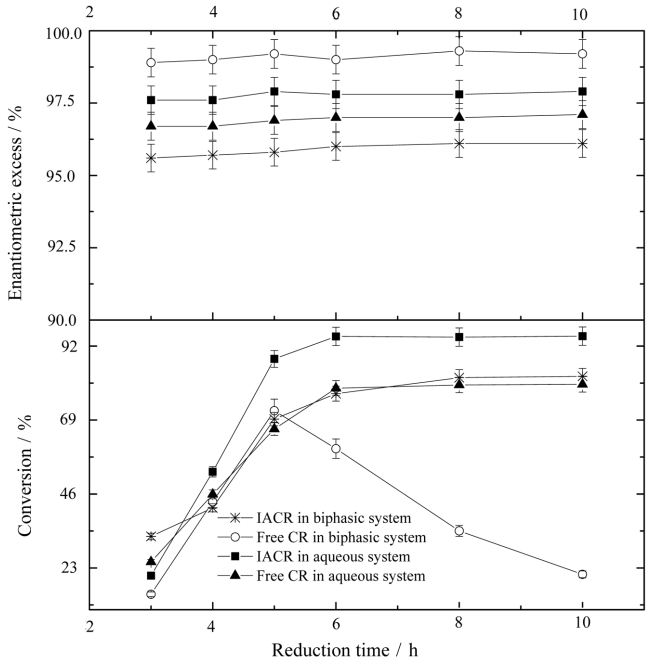


**Fig. 11.** Effect of IACR and free CR on conversion.

S-Licarbazepine was synthesized by the asymmetric reduction of oxcarbazepine with IACR in a toluene/Tris-HCl biphasic system. The effect of the reaction conditions on the reduction of oxcarbazepine was investigated. The volume ratio of toluene and Tris-HCl buffer affected the conversion and enantiomeric excess of S-licarbazepine. The conversion and enantiomeric excess of S-licarbazepine changed when the volume ratio of toluene and Tris-HCl buffer was high or low because the structure of IACR was partially changed. The conversion, the enantiometric excess of S-licarbazepine, and the activity of IACR were influenced by the concentration of S-licarbazepine in the Tris-HCl buffer. The concentration of S-licarbazepine in the Tris-HCl buffer decreased with the increase of the volume ratio of toluene and the Tris-HCl buffer. The conversion decreased with the increase of the S-licarbazepine concentration in the Tris-HCl buffer; thus, the activity of IACR was inhibited by the S-licarbazepine in the Tris-HCl buffer. Therefore, the conversion increased with the increase of the value of $V_o/V_T$ when the value of $V_o/V_T$ was lower than 12.5:10. The changes of conversion were not obvious when the value of $V_o/V_T$ was above 12.5:10. The stereoselectivity of IACR was also affected by the concentration of S-licarbazepine in the Tris-HCl buffer. The enantiometric excess of S-licarbazepine reached 98.6% when the value of $V_o/V_T$ was above 12.5:10. The optimal volume ratio of toluene and Tris-HCl buffer was 12.5:10. NADH dissolved in the Tris-HCl buffer is the coenzyme in the asymmetric reduction of oxcarbazepine. An optimal NADH concentration results in an improved conversion. Ethanol and ethanol dehydrogenase were added to the reaction mixture for the regeneration of NADH. The optimal conditions for the reduction were as follows:

0.08 mol/l NADH, 6% ethanol as a co-substrate, and 0.2 mg of ethanol dehydrogenase (≥300 U/mg). The conversion decreased as the oxcarbazepine concentration increased. The optimal oxcarbazepine concentration was $3.97 \times 10^6$ nmol/l. IACR was suitable for the synthesis of S-licarbazepine in the toluene/Tris-HCl biphasic system. IACR could be reused efficiently five times. The conversion and enantiometric excess of S-licarbazepine were 95.6% and 98.6%, respectively.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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