Enzymatic Resolution of (*S*)-(+)-Naproxen in a Trapped Aqueous–Organic Solvent Biphase Continuous Reactor

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Abstract: A trapped aqueous–organic biphase system for the continuous production of (*S*)-(+)-2-(6-methoxy-2-naphthyl) propionic acid (Naproxen) has been developed. The process consists of a stereoselective hydrolysis of the racemic Naproxen methyl ester by *Candida rugosa* lipase in a trapped aqueous–organic biphase system. The reaction has been carried out in a laboratory-scale continuous-flow stirred tank reactor (CSTR). The staring material has been supplied in and remaining substrate recovered by organic phase. YWG–C_6H_5, a poorly polar synthetic support, has been employed to immobilize the lipase and to restrict the aqueous phase. Lipase immobilized on YWG–C_6H_5 containing aqueous phase has been added into the CSTR to catalyze the hydrolysis. A dialysis membrane tube containing a continuous flow closed-loop buffer has been applied in the CSTR for the extraction of product and recruiting of the aqueous part consumed. Various reaction conditions have been studied. The activity of immobilized enzyme was effected by the polarity of support, the substrate concentration, logP value of organic phase and the product inhibition. At steady-state operating conditions, an initial conversion of 35% has been obtained. The CSTR was allowed to operate continuously for 60 days at 30°C with a 30% loss of activity. The hydrolysis reaction yielded (*S*)-(+)-Naproxen with >90% enantiomeric excess and overall conversion of 30%. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* **68**: 78–83, 2000.

Keywords: Naproxen; lipase; stereoselective hydrolysis; trapped aqueous–organic biphase system; immobilized enzyme; continuous process

INTRODUCTION

(*S*)-(+)-2-(6-Methoxy-2-naphthyl)propionic acid (Naproxen) is a nonsteroidal antiinflammatory drug that belongs to the family of 2-aryl propionic acid derivatives, it is widely used as a drug for human connective tissue diseases. The physiological activity of the *S* form of Naproxen is 28-fold that of the *R* form (Margolin, 1993). Hence, only the *S* form is used as a drug for humans.

Naproxen can be resolved by chemical means, enzymatic means, and asymmetric synthesis. Many enzymatic resolutions are reported with lipase-catalyzed hydrolysis or esterification (Gu et al., 1986; Shau-Wei Tsai et al., 1996).

The conventional method for kinetic resolution of Naproxen is hydrolysis in an aqueous system. Although the reactions mostly run to completion because of high relative concentration (55.5 mol/L) of water, this approach has several drawbacks (Akita et al., 1988; Kise et al., 1991; Caron et al., 1993): Firstly, substrate solubilities are usually low, resulting in low enzymatic reaction rate. Secondly, nonselective chemical background hydrolysis of the ester may occur in water, leading to a lower enantiomeric purity of the reaction product. A third disadvantage of water as solvent may in some cases be the difficulty to work up the reaction product from an aqueous system.

An alternative to medium for Naproxen ester hydrolysis is an aqueous–organic biphase system. Such a system overcomes the problem of ester solubility and remains irreversible because of the presence of a lipid–water interface. However, it shows that it may be not an appropriate system to perform a kinetic resolution: Reactor design for the use of biocatalysts in biphase system presents several complications compared with simple aqueous or organic solvent systems. Agitation conditions may affect the interfacial area between the two phases, by which the reaction rate may be affected. On the other hand, the formation of an emulsion in an aqueous–organic biphase system may complicate workup (Halling, 1987).

Ezio et al. presented a method to continuously produce (*S*)-Naproxen in a column reactor (Ezio et al. 1991). However, the method needed a liquid substrate, and product was present with remaining substrate in the eluate, which may also complicate workup.

According to Halling (Halling, 1987), when the aqueous–organic biphase system has such low proportions of aqueous phase that they are essentially restricted to the pores or interior of solid particles, the term *trapped aqueous–organic biphase* system was used to distinguish the emulsion aqueous–organic biphase system.

In this paper, a trapped aqueous–organic biphase system had been alternated for Naproxen ester hydrolysis. To increase the activity and stability of lipase and to prevent the formation of an emulsion in aqueous–organic biphase system, a YWG−C_6H_5 support has been used to immobilize the lipase and to restrict the aqueous phase to its pores or in interior. YWG−C_6H_5 is poorly polar, high specific surface (100 m^2/g), amorphous multiporous silica on which the

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$-CH_2CH_2C_6H_5$ groups have been bonded. In order to overcome the product inhibition and to facilitate the product recovery, a dialysis membrane tube containing a continuous-flow closed-loop phosphate buffer solution has been applied in the CSTR. Such a system overcomes the problems that agitation conditions may affect the interfacial area between the two phases and emulsion formation in an aqueous–organic biphase system. Because the aqueous part consumed by the hydrolysis may be recruited from the dialysis membrane tube, the thermodynamic reaction equilibrium is favorable. The reaction system offers several advantages for industrial applications. First, because the high specific surface support at which the lipase was immobilized and the aqueous phase was trapped offers a high lipid–aqueous interface, under mild agitation, the support may be suspended and the highest reaction rate may be obtained. Secondly, because the aqueous phase was restricted to the pores or in interior of support, the problem of emulsion formation may be overcome. Thirdly, because the products were extracted by the continuous-flow closed-loop phosphate buffer solution contained in dialysis membrane tube, the product inhibition effecting the activity and stability may be overcome and the product may be easily separated. Because the continuous-flow closed-loop buffer solution compensates the water consumed by the hydrolysis reaction, the thermodynamic reaction equilibrium is favorable.

In this paper, a simpler continuous-flow stirred tank reactor was designed for the trapped water–organic biphase system. The starting material was added, and remaining substrate recovered from the organic phase. The products were extracted by the continuous-flow closed-loop phosphate buffer solution contained in dialysis membrane tube. Various reaction conditions have been studied.

MATERIALS AND METHODS

Materials

Lipase from *Candida rugosa* (780 U/mg) was purchased from Sigma Chemical Co. (±)-Naproxen was purchased from international pharmaceutical factory (Shanghai, China). YWG−C_6H_5, YWG−CN, and YWG−NH_2 were purchased from Chinese Tianjing Chemical Reagent Co. They are amorphous multiporous silicas on which the $-CH_2CH_2C_6H_5$, $-CH_2CH_2CN$, and $-CH_2CH_2NH_2$ groups have been bonded, respectively. The carbon contents of all the supports are close to 10%. The grain size of all the supports is about 40 μm, and the aperture of all the supports is about 30–100 nm. Dialysis membrane (DM 27 × 100 CLR) (MW cutoff: 5,732 Da) was purchased from the SINO-American Biotechnology Company. All other reagents used were obtained from commercial suppliers and were of reagent grade.

Immobilization of Lipase from *C. Rugosa*

A 0.5-g amount of support was suspended in 25 mL of isooctane, and then 0.5 mL of phosphate buffer (0.2 *M*, pH 7.0) containing 75 mg of lipase was added with mild magnetic stirring, after adsorption (30 min at room temperature), the isooctane was decanted.

Enzyme Assays

The hydrolysis of Naproxen methyl ester has been used as a standard assay. The enzyme (75 mg in the native form, 1.075 g in the immobilized form) containing 0.5 mL of phosphate buffer (0.2 *M*, pH 7.0) was added to 25 mL of organic solvent (the concentration of Naproxen methyl ester was 10 mg/mL), the mixture was stirred at 30°C and 150 rpm for 24 h. Samples were taken at 1-h intervals from 0–12 h and then at 24 h for analysis. The activity was calculated by the initial rate of the hydrolysis.

Enzymatic Hydrolysis of (*R, S*)-Naproxen Methyl Ester in a Batch Reactor

Experiments were performed in a 50-mL flask containing 25 mL of organic solution (the concentration of Naproxen methyl ester was 10 mg/mL), the reaction had been started by adding 1.075 g of immobilized enzyme containing 0.5 mL of phosphate buffer (0.2 mol/l, pH 7.0). The mixture was stirred at 150 rpm and 30°C for different times.

Enzymatic Hydrolysis of (*R, S*)-Naproxen Methyl Ester in a CSTR

The scheme of the enzymatic bioreactor is shown in Fig. 1. The immobilized enzyme (225 mg of *C. rugosa* lipase on 1.5 g of YWG−C_6H_5) containing 1.5 mL of phosphate buffer (0.2 *M*, pH 7.0) was added to a laboratory-scale temperature-controlled (30°C) stirred tank reactor (250 mL). The reactor was fed with 75 mL and then a flow (1.0 mL/h) of the organic solvent (the concentration of Naproxen methyl ester was 10 mg/mL). The organic solvent containing the remaining Naproxen methyl ester was eluted out of the reactor. Because of very low solubility (0.062 m*M*, 37°C) of Naproxen in isooctane (Shau-Wei Tsai et al., 1994), no Naproxen was found in the organic eluate. The reactor was equipped with dialysis membrane tube (length, 1 m; flat width, 5 mm) containing a continuous-flow closed-loop buffer solution (0.2 *M*, pH 7.0). The phosphate buffer in the dialysis membrane tube was fed at a flow rate of 120 mL/h through the stirred tank reactor to extract the product. The solubility limit of Naproxen was 4.0 g/L in phosphate buffer solution at 30°C. The phosphate buffer solution containing Naproxen and methanol was eluted out of the reactor and continuously pumped into a separation column packed with active carbon (10 g), and the column was connected in series with the reactor and used to adsorb Naproxen. The Naproxen product extracted into the continuous-flow closed-loop buffer solution can be adsorbed at this stage of the process. After Naproxen was adsorbed, the phosphate buffer solution was recycled into the reactor. The overall volume of recycled phosphate buffer solution was 1,500

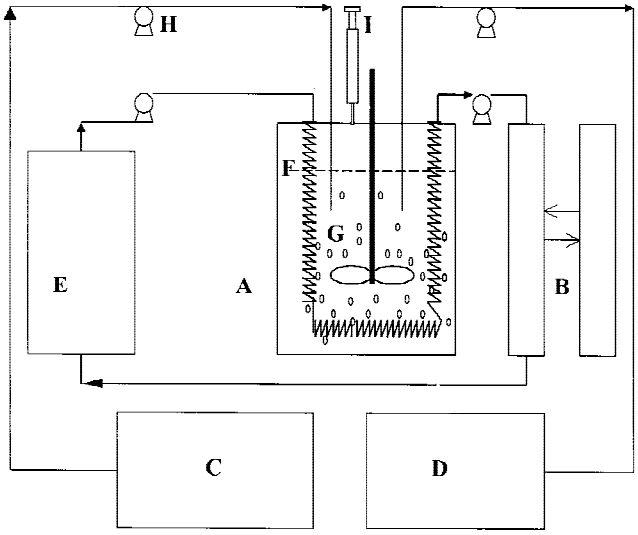

Figure 1. Schematic representation of the CSTR equipped with dialysis membrane containing continuous-flow phosphate buffer. (A) Stirred tank reactor added YWG−C_6H_5 immobilized enzyme; (B) adsorption column packed with active carbon; (C) substrate reservoir; (D) remaining substrate reservoir; (E) buffer reservoir; (F) dialysis membrane tube; (G) enzyme-immobilized on YWG−C_6H_5; (H) pump; (I) air pump.

mL. When the active carbon reached saturation, the column was disconnected and replaced by a similar one without interrupting the flow or conversion of the substrate. A purge was necessary when the concentration of methanol exceed 0.5%.

The adsorption column was regenerated by washing out the products with methanol and roasting at 120°C. The washed column was re-equilibrated with the phosphate buffer solution and then reconnected in series with the stirred tank reactor. Two adsorption columns were used alternatively to make the system continuous.

The methanol solution coming from each washing of the adsorption columns was evaporated at reduced pressure, the precipitated of (*S*)-(+)-Naproxen was separated by filtration and dried.

Analytical Procedure

The enantiomeric excess value of Naproxen was determined by HPLC (HP1090) using a chiral column (Chirex R-NGLY & DNB, Phenomenex) capable of separating the *R* and *S* -isomers of Naproxen. The mobile phase was methanol solution (0.03 *M* ammonium acetate), at a flow rate of 1.0 mL/min.

The enantiomeric excess value of Naproxen methyl ester were determined by HPLC (HP1090) using a chiral column [coated with cellulose tris(3,5-dimethylphenyl carbamate)] capable of separating the *R* and *S* -isomers of Naproxen ester. The mobile phase was a mixture of 99.5% *n*-hexane/0.5% 2-propanol (v/v), at a flow rate of 0.5 mL/min. UV detection at 254 nm was used for quantification at the 25°C.

The extent of conversion of substrate was monitored with a UV spectrophotometer (UV-120-02, Shimadzu Co.) at 270 nm or calculated by the ee_s and ee_p according to $C = ee_s/(ee_s + ee_p)$ (Chen et al. 1982).

Synthesis of Ester

The methyl ester of (*R, S*)-Naproxen was prepared by the classical methodology (Brook et al., 1983) using thionyl chloride and methanol. Thionyl chloride, 15 ml (0.2 mol), was added dropwise to a cooled, stirred suspension of Naproxen (0.12 mol) in methanol (250 mL). The reaction mixture was refluxed for 2.5 h. the solvent was evaporated, and the residue was purified by column chromatography using SiO_2 as adsorbent and isooctane as eluent.

RESULTS AND DISCUSSION

From an application point of view, adsorption is the most suitable immobilization procedure due to its simplicity and low cost. However, its major drawback is that immobilized enzyme tends to be desorbed from the support during utilization of the biocatalyst. However, this phenomenon does not occur in trapped aqueous–organic media. One advantage restricted to the trapped aqueous–organic solvent biphase system is that the enzyme may be effectively immobilized on or within support. Since the lipase will not dissolve in the organic phase, it will remain in the aqueous phase, and this in turn will be retained by the support. This "free immobilization" may assure that 100% lipase has been immobilized (Halling, 1987). In this paper, using isooctane as the reaction medium, the lipase from *C. rugosa* was immobilized by the adsorption on different synthetic supports (YWG−C_6H_5, YWG−CN_5 and YWG−NH_2). The immobilization of the enzyme was essentially complete within 30 min at room temperature, the isooctane was decanted, and the activity of lipase was not found in the supernatant thereafter.

In this paper, we selected YWG−C_6H_5 as the support of immobilized enzyme. This support presents several advantages: (1) It is commercially available in large quantities. (2) Its high specific surface and hydrophobicity allow high protein loading by adsorption (136.4 mg lipase/g support in aqueous medium). The adsorptive power was so strong that lipase was effectively immobilized (and cannot be eluted by solutions containing 1 mol/L $(NH_4)_2SO_4$ or 25% ethanol). On the other hand, the high specific surface of the support allows the trapped aqueous shell that surrounds the support to produce a high lipid–aqueous interface with the organic solvent. (3) It is a hydrophobic support that has good affinity toward the Naproxen methyl ester, and a local increase of the substrate concentration around the enzyme on the support result in the enhancement of lipase activity. The activities of hydrolysis of Naproxen methyl ester catalyzed by the free lipase and the lipase immobilized on YWG−NH_2, YWG−CN, and YWG−C_6H_5 in the batch reaction were 0.2, 0.64, 1.40, and 2.10 $\mu M \cdot mg^{-1} \cdot h^{-1}$, respectively. (4) Since the support has some hydrophilic

−OH group that was not coated by the hydrophobic $-CH_2CH_2C_6H_5$ group, the aqueous phase may be retained by the support. When the lipase was immobilized by the adsorption on the synthetic supports (YWG−C_6H_5) using isooctane as immobilization medium, the water phase containing lipase was restricted and trapped to the support. The immobilization of the enzyme was essentially complete within 30 min at room temperature. When the isooctane was decanted, a water-free phase and the activity of lipase were not found in the supernatant thereafter.

Using different concentrations of Naproxen methyl ester in the batch reaction, some experimental data of the conversion and yield with Naproxen methyl ester concentration as the parameter are shown in Fig. 2, in which a decreasing conversion with increasing Naproxen methyl ester concentration was found. However, the maximum yield occurred when the Naproxen methyl ester concentration was 10 mg/mL. There was still no evidence to prove the real role of increasing Naproxen methyl ester concentration above 10 mg/mL on decreasing the yield of Naproxen. Thus, further study on the possibility of substrate inhibition was necessary.

The products of the hydrolysis of Naproxen methyl ester, Naproxen and methanol, may inhibit or inactivate the lipase that interconverts it. Because the trapped aqueous phase containing lipase was restricted to the pores or in interior of the support in the system, this could provide an environment to dissolve some Naproxen, and methanol acted as an inhibitor to the enzyme. By the batch reaction, the methanol and Naproxen were tested independently to determine their effects on the activity of the enzyme. Addition of various amounts methanol at the beginning of a hydrolysis reaction resulted in observable reduction in enzyme activity (Fig. 3). However, addition of various amounts (*S*)-Naproxen (from 0.5 to 1.5 g) at the beginning of a hydrolysis reaction did not result in an observable reduction in enzyme activity (data not shown). This result may be explained by following facts: (1) The lower solubility of Naproxen in the aqueous phase, to which the enzyme was exposed, did not result in observable inhibition to the activity of immobilized lipase. (2) Insoluble Naproxen that was produced precipitated in the aqueous phase, which as a support of immobilized lipase may increase the reaction interface.

If starting material (Naproxen methyl ester) can be supplied in and all products (Naproxen and methanol) recovered from the organic phase, then it is possible to construct a simple continuous reactor. However, the low solubility of methanol and Naproxen in isooctane is an important factor, which limits the application of a continuous-flow reactor to the hydrolysis of the Naproxen ester with immobilized lipase. Due to the low solubility of Naproxen in isooctane (0.062 m*M*, 37°C) (Shau-Wei Tsai et al., 1994) and in water (17.391 m*M*, 35°C, phosphate buffer, pH 6.5, 0.1 *M*) (Ezio et al., 1991), an insoluble Naproxen precipitate is found in the aqueous phase. In the biphase system, the precipitation of the product would make its recovery uneasy and cumbersome. To overcome these limitations, we attempted to use the organic medium with a solvent hydrophobicity low than that of isooctane. Decreasing the hydrophobicity of solvent resulted in the enhancement of solubility of Naproxen, however, at the price of decreasing the enzyme activity. Decreasing the solvent hydrophobicity resulted in a decrease in enzyme activity. In Table I the activity of the

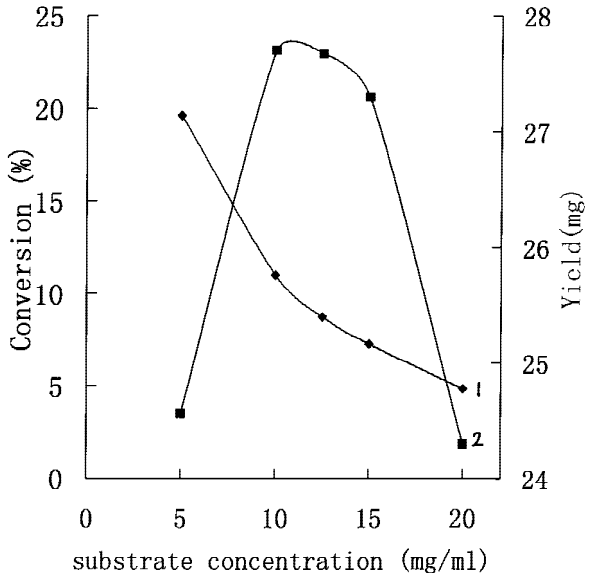

Figure 2. Effect of substrate concentration on conversion and yield. Reactions were started by added 1.075 g of immobilized lipase on YWG−C_6H_5 containing 0.5 mL of phosphate buffer (0.2 *M*, pH 7.0) into 25 mL of isooctane with different substrate concentration. After reaction 24 h at 30°C and 150 rpm. Samples were taken from the organic and aqueous phases for analysis. The degree of conversion of the reaction was calculated by the ee_s and ee_p according to $C = ee_s/(ee_s + ee_p)$ (Chen et al. 1987). (1) Conversion; (2) yield.

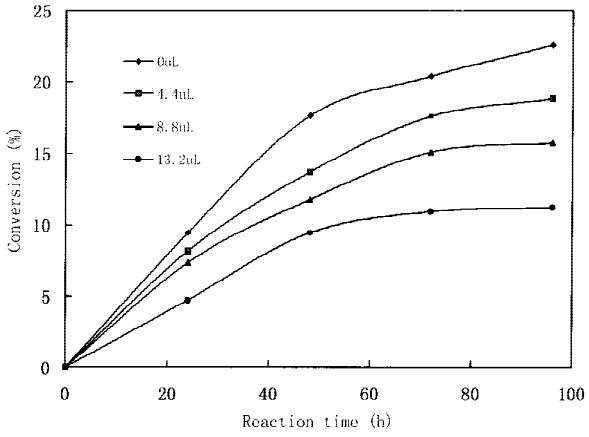

Figure 3. Effect of methanol on the activity of immobilized enzyme. Experiments were performed in a 50 mL flask containing 25 mL of isooctane solution (the concentration of Naproxen methyl ester was 10 mg/mL), various amounts methanol (0–13.2 μL) were added into the reaction system at the beginning of a hydrolysis reaction, respectively. The reaction had been started by added 1.075 g of immobilized enzyme on YWG−C_6H_5 containing 0.5 mL of phosphate buffer (0.2 *M*, pH 7.0). Samples were taken from organic phase and aqueous phase at 24-h intervals from 0 to 96 h for analysis.

Table I. Effect of organic solvent on activity of enzyme and solubility of Naproxen.

Solvent	Solvent hydrophobicity[a] (log *P*)	Concentration of saturated Naproxen (mmol/L)	Relative activity (%)	*C* (%)	ee_p (%)	*E*
Isooctane	4.5	0.062[b]	100.0	42.5	92.3	50
Isooctane/toluene						
95/5 (mol/mol)	4.4	1.30	95.0	40.4	92.8	51
90/10 (mol/mol)	4.3	2.55	90.0	38.3	93.4	53
85/15 (mol/mol)	4.2	3.79	86.5	36.8	91.2	37
80/20 (mol/mol)	3.9	5.00	81.2	34.5	91.5	41
Hexane	3.5		77.6	33.0	89.1	26
Cyclohexane	3.2		75.0	31.9	88.5	25
Toluene	2.5		8.6	3.7	93.3	29
Acetone	−0.23		0.0			

[a]Values of log *P* are defined as the logarithm of the partition of a given solvent between *n*-octanol and water. Values of log *P* from 3.9 to 4.5 for the mixture were calculated as follows: $\log P = (1 - x) \log P_{\text{toluene}} + x \log P_{\text{isooctanes}}$, where *x* is the mole fraction of isooctane (Lanne et al., 1987; Reslow et al., 1987; Valivety et al., 1991).

[b]Shau-Wei Tsai et al. (1994).

YWG−C_6H_5 immobilized lipase was shown as a function of log *P*.

If the reaction system includes a flowing aqueous phase into which the methanol and Naproxen partition, then its concentration in the trapped aqueous phase, to which the lipase is exposed, may be kept low. Hence, to prevent the methanol inhibition of the enzymatic activity and the same to remove (*S*)-Naproxen, running the reaction at a flowing bioreactor to remove the methanol and Naproxen and recycling the aqueous phase would be advantageous. However, the flowing aqueous phase may emulsify with the organic phase. In order to overcome the limit, a hydrophilic DM tube containing a continuous-flow closed-loop phosphate buffer solution was applied in the reactor to separate the flowing aqueous phase from the organic phase. The DM was a hydrophilic semipermeable membrane, which allows a molecule having MW lower than 5,000 Da to pass through the membrane. However, the nonpolar isooctane was not allowed to pass through the membrane (because the organic

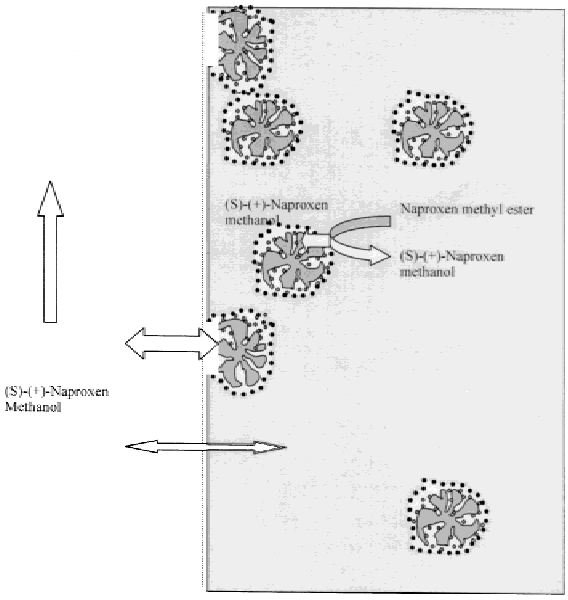

Figure 4. Processes occurring during action of immobilized lipase in the trapped aqueous–organic biphase. (Dark gray areas) Support particle; (●) lipase; (white areas) trapped aqueous phase and continuousflow buffer solution; (light gray areas) organic solvent phase; (·····) dialysis membrane.

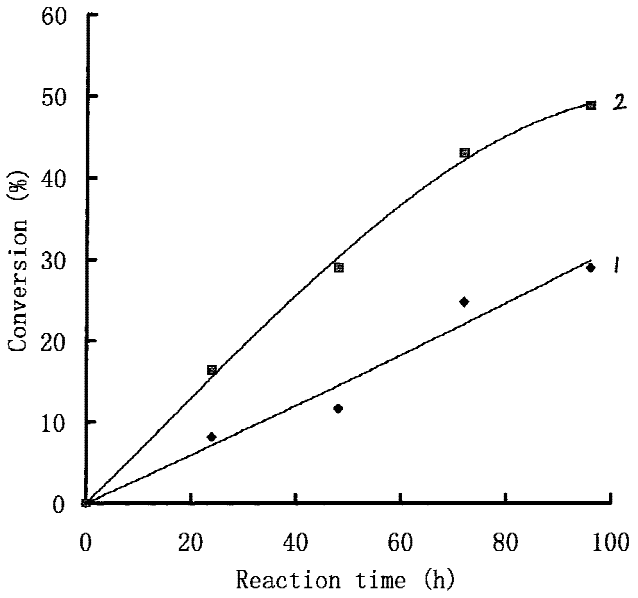

Figure 5. Effect of product extraction on the activity of immobilized enzyme. Experiments were performed in a 250 mL flask equipping with 1 m dialysis membrane tube, 25 mL of isooctane solution (the concentration of Naproxen methyl ester was 10 mg/mL) was added, and the reaction was started by adding 1.075 g of immobilized enzyme on YWG−C_6H_5 containing 0.5 mL phosphate buffer (0.2 *M*, pH 7.0). The flow rates of phosphate buffer contained in the dialysis membrane tube were 0 and 120 mL/h, respectively. Samples were taken from organic phase at 24-h intervals from 0 to 96 h for analysis. The extent of conversion of substrate was monitored by UV spectrophotometer (UV-120-02, Shimadzu Co.) at 270 nm. (1) Flow rate of phosphate buffer: 0 mL/h. (2) Flow rate of phosphate buffer: 120 mL/h.

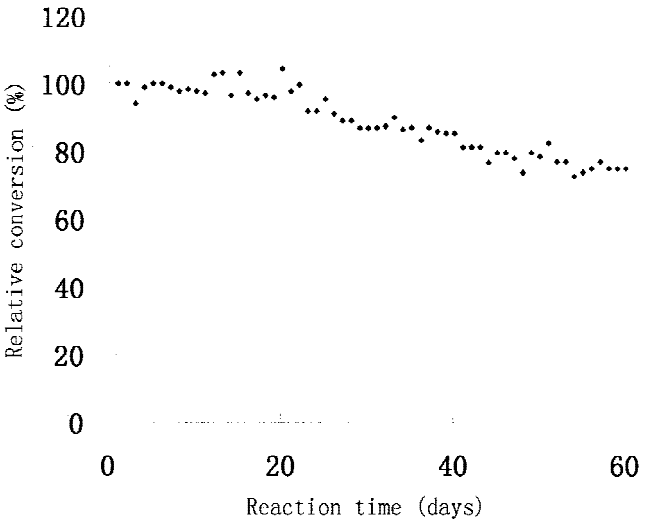

Figure 6. Operating stability of immobilized lipase in the CSTR equipped with dialysis membrane containing continuous-flow phosphate buffer. At steady state, the flow rate of organic phase was 1 mL/h, and the flow rate of continuous-flow closed loop phosphate buffer was 120 mL/h. The stability was obtained by measurement conversion.

phase does not wet the membrane material and so faces adverse capillary pressures). In the reactor, the lipase immobilized was adsorbed and desorbed ceaselessly on the DM tube exterior when the agitation speed closed to 150 rpm. As shown in Fig. 4, when the immobilized enzyme suspended in the organic phase was adsorbed by the DM surface, the water shell around the support was confronted with the water shell restricted on the exterior surface of dialysis membrane tube. The Naproxen and methanol were extracted into the intracavity of the dialysis membrane tube and recovered from the continuous-flow closed-loop phosphate buffer solution. Another possible mechanism may be that the products that existed in isooctane were extracted directly into the aqueous phase restricted in the intracavity of the dialysis membrane tube.

Substrate hydrolysis is now a factor of diffusion for both the immobilized lipase and the tubing used for phosphate buffer circulation. Agitation and flow conditions may affect the hydrolysis rate of substrate. A higher agitation speed and flow rate are needed in the reaction system, either to keep the immobilized lipase suspended or to keep the product extracted. As the agitating speed increases beyond 150 rpm, and the flow speed of phosphate buffer within dialysis membrane tube increases beyond 120 mL/h, no obvious increase of conversion of substrate has been found. So the diffusion limit for the immobilized lipase and the extraction limit for the tubing used for phosphate buffer circulation can be overcome.

By the extraction of continuous-flow closed-loop phosphate buffer solution, the product concentration in the trapped aqueous phase in which the lipase existed may be kept low and the inhibition of methanol may be overcome. This would be advantageous to the reaction. Compared with the hydrolysis reaction without extracting products, the conversion was significantly enhanced (Fig. 5).

At steady-state operating conditions, an initial conversion of 35% has been obtained. The reactor has been operated continuously for 60 days at 30°C with a 30% loss of activity (Fig. 6). The hydrolysis reaction yielded (*S*)-(+)-Naproxen with >90% enantiomeric excess and overall conversion of 30%.

By the bioreactor, (*S*)-(+)-Naproxen with >90% enantiomeric excess may be obtained from the continuous-flow aqueous phase, and remaining Naproxen methyl ester may be recovered from the organic phase. The immobilized enzyme is permanently remained in the reactor. In conclusion, the enzymatic process described in this work can be readily scaled up for industrial application since it is highly efficient, yields high optically pure (*S*)-(+)-Naproxen, separates it from the remaining substrate at the same time, and withstands long-term operation.

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