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Enzymatic production of enantiopure ketoprofen in a solvent-free two-phase system

Ju Nam Jin [a], Sang Hyun Lee [a], Sun Bok Lee [a,b,*]

[a] *Department of Chemical Engineering, Pohang University of Science and Technology, San 31, Hyoja-Dong, Pohang 790-784, South Korea*
[b] *Division of Molecular and Life Sciences, Pohang University of Science and Technology, San 31, Hyoja-Dong, Pohang 790-784, South Korea*

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Abstract

Enzymatic hydrolysis conducted in a medium composed of solely substrate is considered to resolve racemic ketoprofen esters. In a system composed of two components, the pure liquid substrate (organic phase) and water (aqueous phase), hydrolysis products can be efficiently removed from the reaction mixtures. Accordingly, in this study we designed a solvent-free two-phase system for the enantioselective enzymatic hydrolysis of ketoprofen esters. In order to further optimize this system, the influences of various factors, such as the pH of the aqueous phase, temperature, enzyme content, and the alcohol chain length of esters, were examined on conversion and enantiomeric excess. 1N $NaHCO_3$ was identified as the most efficient aqueous phase for the extraction of ketoprofen. Changes in the amount of enzyme did not significantly affect the maximum conversion or the enantiomeric excess. On the other hand, ketoprofen esters with shorter alcohol chains displayed higher initial reaction rates and conversions in solventless media. In the case of ketoprofen propyl ester, for example, the productivity of the solvent-free two-phase system was about 10–100 times higher than that obtained to date for ketoprofen esterification with alcohols in organic solvents. The enantioselectivities obtained in solvent-free media were similar to those obtained for the enantioselective esterification of ketoprofen in organic solvents.

Keywords: Solvent-free system; Two-phase system; Ketoprofen; Hydrolysis; Lipase; Enantioselectivity

1. Introduction

Enzymatic catalysis in non-aqueous solvents has attracted considerable interest in recent years as an efficient approach to the synthesis of natural products, pharmaceuticals, fine chemicals and food ingredients. Under non-aqueous conditions, the industrial utility of enzymes can be improved because of enhanced enzyme thermostability, ease of product and enzyme recovery, and the ability to catalyze reactions that are kinetically or thermodynamically unfavorable in aqueous solutions [1]. However, it would be technically beneficial if enzymatic reactions were performed in mixtures of substrates in the absence of bulk solvents. Enzyme-catalyzed reactions in solvent-free media have been described in recent years [2–8]. This approach can combine the advantage of non-aqueous enzymology with high levels of productivity. Another advantage of the solvent-free system is its lack of solvent, which minimizes the environmental impact and

* Corresponding author. Tel.: +82-54-279-2268; fax: +82-54-279-2699.
E-mail address: sblee@postech.ac.kr (S.B. Lee).

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the reaction volume, thus reducing the size of the equipment required and the associated capital costs.

Among the racemic drugs, the 2-arylpropionic acids (the "profen" family) constitute an important group of non-steroidal anti-inflammatory drugs (NSAIDs), which are widely used as racemic mixtures to control the symptoms of arthritis and related connective tissue diseases. The majority of synthetic chiral drugs are now marketed as racemates [9], but this situation is rapidly changing due to FDA regulations and recent advances in biocatalytic methods. The kinetic resolution of racemic compounds using the stereoselectivity of enzymes is regarded as a valuable method of obtaining chiral compounds of high optical purity [1–4,9–11]. In case of ketoprofen (2-(3-benzoylphenyl)propionic acid), which is one of the NSAIDs, the (*S*)-enantiomer reduces inflammation and relieves pain, whereas the (*R*)-enantiomer can be used as a toothpaste additive to prevent periodontal disease. Therefore, efforts are being made to obtain optically pure ketoprofen [12–15].

It has been shown that lipases can catalyze, with good yields, the resolution of racemic ibuprofen in solventless media [4,16,17]. However, the enzymatic resolution of ketoprofen by lipases in solvent-free media has been unsuccessful. This has been mainly ascribed to the spontaneous non-enzymatic esterification of ketoprofen under solvent-free high substrate loading. If such non-specific chemical reactions are significant, the enantiomeric excess of the required product can be markedly reduced.

In this work, we developed a novel solvent-free two-phase system for the enzymatic resolution of ketoprofen. This solvent-free two-phase system offers the merits of both solvent-free systems and two-phase systems. The main advantage of a solvent-free system is that the enzymatic reaction can be performed at very high substrate concentrations. For example, we were able to carry out the lipase-catalyzed kinetic resolution of ketoprofen esters at concentrations as high as 4.1 M, which is the highest substrate concentration employed for the resolution of ketoprofen. In addition, due to the use of a two-phase system, the recovery of both product and biocatalyst is easier. Enantiopure ketoprofen can be prepared by chemical hydrolysis of the product remaining in the organic phase after enzymatically resolving the racemic mixtures in a solvent-free two-phase system.

2. Materials and methods

2.1. Materials

Novozym 435 (*Candida antarctica* type B lipase immobilized on acrylic resin) and Lipozyme IM (*Rhizomucor miehei* lipase immobilized on anion-exchange resin) were provided by Novo Nordisk (Bagsvaerd, Denmark). Lipase OF (*Candida rugosa* lipase) was purchased from Meito Sangyo (Tokyo, Japan), (*R*,*S*)-ketoprofen from Kuk Jeon (Seoul, Korea), and (*S*)-ketoprofen from Sigma (St. Louis, USA). Silica gel (particle size: 40–63 μm) was purchased from Merck (Darmstadt, Germany). All other chemicals used in this work were of analytical grade and were used without further purification.

2.2. Synthesis of ketoprofen esters

Ketoprofen esters were prepared by the spontaneous chemical esterification of racemic ketoprofen with various alcohols. In 50 ml vials, 20 mmol of alcohol was added to 20 mmol of racemic ketoprofen. The mixture was heated on a hotplate with stirring until all substrates liquefied, and then further incubated at 90 °C in a water bath. To produce the methyl, ethyl, and propyl esters of ketoprofen, the reaction temperature was lowered to 60 °C in order to reduce the evaporation of alcohols during the reaction. Products were purified by flash chromatography using a column packed with silica gel (particle size: 40–63 μm), with *n*-hexane and ethyl acetate (16:1) as eluent. To analyze each eluent fraction, thin layer chromatography was performed on 0.25 mm silica gel plates (60F-254, Merck) using a mixture of *n*-hexane and ethyl acetate (4:1) as developing agent and *p*-anisaldehyde solution (1.5% in ethanol) as visualizing agent. After purification of products, the collected pool was evaporated in a rotary evaporator at 60 °C. The authenticity and purity of the ketoprofen esters were determined by ^{1}H NMR and HPLC, respectively.

2.3. Enzymatic hydrolysis of ketoprofen esters in a solvent-free single-phase system

To carry out the enzymatic hydrolysis of ketoprofen ester in a single-phase system, the enzyme and the substrate were humidified with saturated salt solutions

prior to the enzyme reaction. The water activity (a_w) of the substrate solution (4.9 mmol) and of the enzyme (0.3 g Novozym 435) were controlled by equilibrating each sample at 60 °C for 5 days with a saturated solution of various salts; lithium chloride ($a_w = 0.10$), magnesium chloride (0.30), sodium bromide (0.50), sodium chloride (0.75), and potassium sulfate (0.95) [18]. Enzyme reactions were started by pouring the enzyme into vials containing ketoprofen decyl ester, and placing these vials in a shaking water bath.

2.4. Enzymatic hydrolysis of ketoprofen esters in a solvent-free two-phase system

The enzymatic hydrolysis reactions in a solvent-free two-phase system were conducted, unless otherwise stated, in a vial (50 ml) containing 4.9 mmol of ketoprofen ester, 25 ml of basic aqueous solution (1N $NaHCO_3$), and 0.3 g of enzyme. Enzyme was added to the reaction medium, composed of ketoprofen ester (top phase) and an aqueous solution (bottom phase), and then the reaction mixture was gently shaken in a water bath at 150 rpm. Periodically, 10 μl aliquots were removed from the top and bottom phases, and the reaction conversion and the enantiomeric excess were evaluated, using the methods described in the next section. The liquid volumes corresponding to 4.9 mmol of ketoprofen esters were 1.14, 1.23, 1.30, 1.40, 1.54, 1.81, and 2.00 ml for the methyl, ethyl, propyl, butyl, hexyl, octyl, and decyl ester, respectively, at room temperature.

2.5. Analytical methods

The concentrations of (*R*,*S*)-ketoprofen esters, and of (*R*)- and (*S*)-ketoprofen were measured by HPLC. Separation was accomplished using a Shimadzu HPLC system (Model LC-10A, Japan) equipped with a reverse-phase C_{18} column (Nova-pak, Waters, USA) and a UV detector (254 nm). The mobile phase consisted of acetonitrile/water (80/20, v/v) containing 50 μl phosphoric acid/l. The flow rate was maintained constant at 1.0 ml/min.

The conversion of ketoprofen ester was calculated using the following equation:

$$c = \frac{[W]V_W + [E]V_E}{N_0} \times 100$$

where c is the conversion (%), $[W]$ the ketoprofen concentration in the aqueous phase (mM), $[E]$ the ketoprofen concentration in the organic phase (mM), V_W the volume of the aqueous phase (l), V_E the volume of the ester phase (l), and N_0 the initial amount of ketoprofen ester (mmol). In this equation, the mixing effect on the composition of each phase and the volume change during the reaction were assumed to be insignificant.

Chiral resolution of the ketoprofen enantiomers was accomplished using a HPLC system (Eurochrome 2000, Knauer, Germany) equipped with a chiral column (CHIREX Phase 3005, Phenomenex, USA) and a UV detector (254 nm). The mobile phase was 30 mM ammonium acetate in methanol, and the flow rate was maintained at 1.0 ml/min. Enantiomeric excesses of product ($e.e._p$) and substrate ($e.e._s$), and enantioselectivity (E) were calculated using the following equations:

$$e.e._p\,(\%) = \frac{[(R)] - [(S)]}{[(R)] + [(S)]} \times 100,$$

$$e.e._s\,(\%) = \frac{c}{100 - c} \times e.e._p,$$

$$E = \frac{\ln[1 - c(1 + e.e._p)]}{\ln[1 - c(1 - e.e._p)]}$$

where $[(R)]$ and $[(S)]$ represent the concentrations of the (*R*) and (*S*) forms of ketoprofen and c the reaction conversion (%).

3. Results and discussion

3.1. Design of a novel biocatalytic process for the kinetic resolution of ketoprofen

Initially, we tried to develop an esterification process to produce (*S*)-ketoprofen in a solvent-free system. For this purpose, the esterification of ketoprofen with decanol was carried out without any solvent at 70 °C using Novozym 435 as a biocatalyst. However, when the same reaction was carried out in the absence of a biocatalyst, a significant amount of ketoprofen decyl ester also formed spontaneously. This result is in accordance with an earlier report [13], where the esterification reaction between dodecanol and ketoprofen was studied in solvent-free media.

We further examined the spontaneous esterification of ketoprofen with various alcohols. It was found that the alkyl esters of ketoprofen could be synthesized in good yields by incubating the substrate mixtures without catalyst. For example, when 5 mmol of ketoprofen was incubated with 10 mmol of decanol at 70 °C for 120 h, 82% of the ketoprofen was converted to ketoprofen decyl ester. The extent of spontaneous chemical esterification was enhanced by increasing the temperatures. This indicated that the enantiomeric excess of the product could be drastically reduced by non-specific chemical esterification in solvent-free media. Therefore, we needed to develop a biocatalytic process in which spontaneous chemical reaction could not occur under solvent-free, high substrate loading conditions.

To explore the possibility of conducting hydrolysis in solvent-free media, we undertook the lipase-catalyzed hydrolysis of ketoprofen decyl ester in a single-phase. Reactions were carried out in a wide range of water activities by equilibrating the substrate and the enzyme with various saturated salt solutions. Conversion increased on increasing the water activity of the reaction medium (data not shown). In addition, spontaneous hydrolysis of the ketoprofen decyl ester was not observed when it was incubated for 8 days at 70 °C in the presence of excess water. These observations implied that excess water did not impair the efficiency of the system. In general, the equilibrium of enzymatic hydrolysis in organic media is very unfavorable to the hydrolytic direction, and hence conversion can be enhanced if products are removed from reaction mixtures. This can be done by extracting the product into a water phase. Based on these observations, a solvent-free two-phase system was designed for the enzymatic hydrolysis of ketoprofen esters (Fig. 1).

3.2. Aqueous phase composition of a solvent-free two-phase system

The composition of the aqueous phase is important, since conversion can be enhanced if the products are properly extracted from the organic phase to the aqueous phase. In the case of hydrolysis of the ketoprofen esters, the reaction products are ketoprofen and alcohols. Due to the acidic nature of ketoprofen, ketoprofen is deprotonated under alkaline conditions, and thus the (*R*)-ketoprofen produced during the reaction

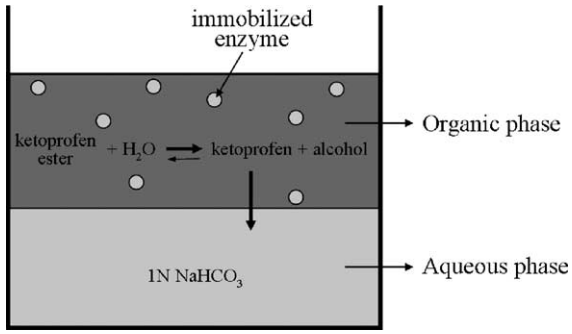

Fig. 1. Schematic diagram of the solvent-free two-phase system for the hydrolysis of ketoprofen esters.

can be effectively transferred to the aqueous phase if aqueous solution is basic. To examine this possibility, the effect of pH in the water phase was investigated on the kinetic resolution of racemic ketoprofen decyl ester (Fig. 2). We found that this extractive reaction system using a basic solution significantly enhanced conversion as compared to that observed for neutral water. However, the conversion was drastically reduced at higher pH. It appears that the use of strong basic solutions as an extractive medium causes the

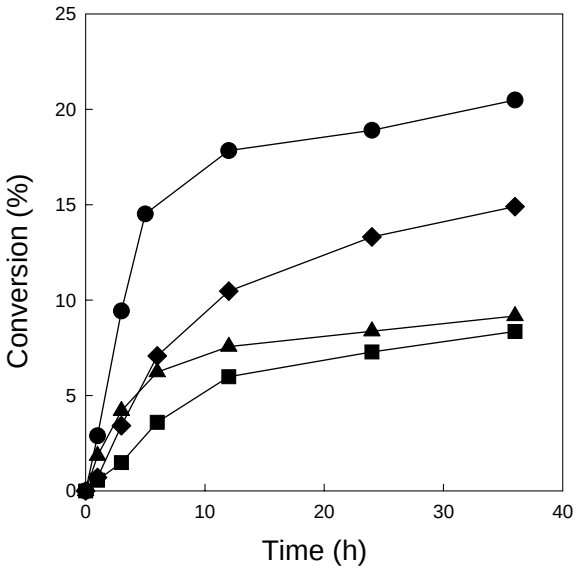

Fig. 2. Influence of the pH of the aqueous phase on the hydrolysis of ketoprofen decyl ester. Reaction conditions: 4.9 mmol substrate, 0.3 g Novozym 435, 70 °C. (■) water (pH 7.0), (●) 1N $NaHCO_3$ (pH 8.7), (◆) 1N Na_2CO_3 (pH 11.2), (▲) 1N NaOH (pH 13.0).

inactivation of the enzyme and the production of undesirable by-products. Since ibuprofen, a structural analog of ketoprofen, had been reported to be racemized by base [19], the possibility of ketoprofen racemization in 1N $NaHCO_3$ solution was also investigated. However, when (*S*)-ketoprofen was incubated in 1N $NaHCO_3$ solution at 70 °C for 48 h, no racemization of (*S*)-ketoprofen was detected. Therefore, 1N $NaHCO_3$ solution (pH 8.7) was used as an aqueous phase for the optimization of reaction conditions. In separate experiments, we confirmed that most of the product ((*R*)-ketoprofen) could be transferred to the aqueous phase (1N $NaHCO_3$ solution). We then studied the influence of various factors, such as temperature, enzyme content, and alcohol chain length of esters, on the conversion and the enantiomeric excess.

3.3. Effect of reaction conditions

To select an enzyme preparation suitable for solvent-free systems, the kinetic resolution of ketoprofen decyl ester was carried out in a two-phase system using three commercial lipases; Novozym 435, Lipozyme IM, and Lipase OF. Of these, Novozym 435 showed the highest reaction rate and conversion. When 0.1 g enzyme was used for the hydrolysis of ketoprofen ester, the conversions after 48 h reaction were 13.8, 8.1, and 7.8% for Novozym 435, Lipozyme IM, and Lipase OF, respectively. Lipase OF had a stereopreference for (*S*)-ketoprofen whereas Novozym 435 and Lipozyme IM showed a selectivity for (*R*)-ketoprofen (data not shown). We chose Novozyme 435, which is selective for (*R*)-ketoprofen and shows a higher initial rate, for the kinetic resolution of ketoprofen decyl ester.

In order to increase the transfer rate of the substrate to the enzyme in a two-phase system, it is desirable to retain both the enzyme and the substrate in the same phase. In the cases of Lipozyme IM and Lipase OF, the enzyme and the substrate were located in the aqueous and organic phases, respectively, and thus the reaction proceeded at the interface. However, Novozym 435, which was immobilized on hydrophobic acrylic resin, remained in the organic phase during the reaction, which may have resulted in the higher reaction rate of Novozym 435.

The influence of the amount of biocatalyst used is shown in Table 1. As expected, the initial reaction rates increased with increasing the amount of enzymes. However, enzyme content did not significantly affect the final conversion (at 72 h) or the enantiomeric excess of the product. In terms of enantioselectivity, best results were obtained with 300 mg of Novozym 435.

Table 1
Influence of the amount of enzyme on the hydrolysis of ketoprofen decyl ester

Novozym 435 (g)	Initial reaction rate (μmol h^{-1})	Conversion (%, at 72 h)	$e.e._s$ (%)	$e.e._p$ (%)	*E*
0.1	34	17.3	12.9	61.7	4.8
0.2	71	21.0	16.5	62.1	5.0
0.3	130	22.5	18.5	63.7	5.4

The reactions were also performed at various temperatures (Fig. 3). Final conversions increased with increasing reaction temperatures to 70 °C, but decreased at 90 °C. In view of the fact that Novozyme 435 has a maximum activity in the range 70–80 °C, thermal inactivation of Novozym 435 may have caused the lower observed conversion at 90 °C than at 70 °C. The enantioselectivity of Novozym 435 was also reduced on increasing the temperature (Table 2). In general, the selectivity of an enzyme is inversely proportional to

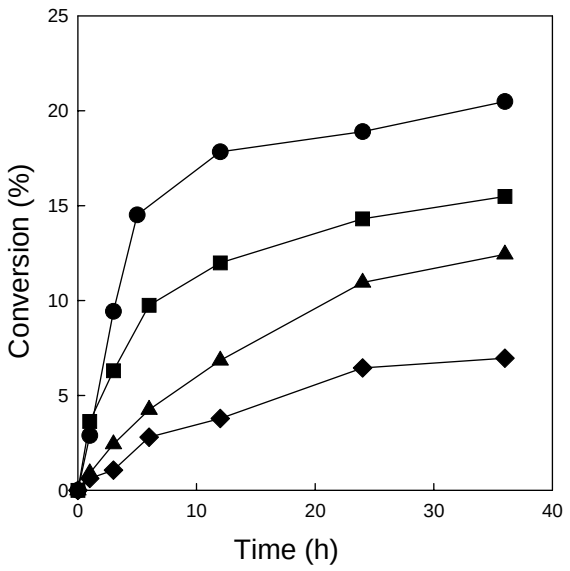

Fig. 3. Time courses of the hydrolysis of ketoprofen decyl ester at different reaction temperatures. Reaction conditions: 4.9 mmol substrate, 0.3 g Novozym 435. (◆) 30 °C, (▲) 50 °C, (●) 70 °C, (■) 90 °C.

Table 2
Influence of temperature on the hydrolysis of ketoprofen decyl ester

Temperature (°C)	Initial rate (μmol h^{-1})	Conversion (%, at 36 h)	e.e.$_s$ (%)	e.e.$_p$ (%)	E
30	19	7.0	5.2	69.1	5.8
50	36	12.4	8.7	61.4	4.6
70	127	20.5	14.9	57.7	4.3
90	180	15.5	10.3	55.9	3.9

the temperature, because the flexibility of enzyme increases with the rise in temperature and hence selectivity decreases [20]. The observed decreased enantioselectivity of Novozym 435 at higher temperatures can be considered to reflect the general case.

The enzymatic hydrolysis of ketoprofen decyl ester was slow in solvent-free media, and thus we examined the use of ketoprofen methyl ester in the solvent-free two-phase system. However, unfortunately, spontaneous hydrolysis of ketoprofen methyl ester occurred at temperatures over 70 °C, and hence the reaction was carried out at 50 °C to minimize unwanted reactions. As shown in Fig. 4, the initial rate and conversion of ketoprofen methyl ester were considerably higher than those of ketoprofen decyl ester. The methanol produced from ketoprofen methyl ester is very water soluble and thus the chemical equilibrium achieved may favor hydrolysis; however, the enantiomeric excess of the product was drastically reduced after 4 h of reaction (Fig. 4).

3.4. Effect of the chain length of the alcohol moiety

Previous experiments on two substrates (ketoprofen methyl ester and ketoprofen decyl ester) indicated that the reactivity of substrates depends on the nature of the acyl donor of the ketoprofen esters. To evaluate the effect of the chain length of alcohol moiety on conversion and enantioselectivity, enzymatic hydrolysis was carried out in a solvent-free two-phase system using the ketoprofen esters with various alcohol chain lengths. The hydrolysis of esters containing short chain alcohol moieties (methyl, ethyl, and propyl ester) showed a higher initial rate and conversion (Fig. 5). Since short chain alcohols produced by the hydrolysis of ketoprofen esters may be quickly dissolved and partitioned into the aqueous phase, the chemical equilibrium is more favorable to the hydrolytic direction. On the other hand, long chain alcohols (e.g., *n*-octanol and *n*-decanol) rarely

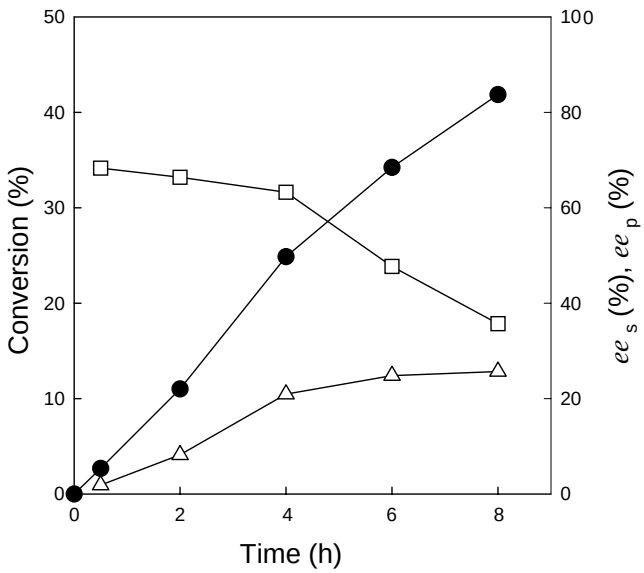

Fig. 4. Hydrolysis of ketoprofen methyl ester in a solvent-free two-phase system. Reaction conditions: 9.8 mmol substrate, 0.1 g Novozym 435, 50 °C. (●) conversion (%), (□) enantiomeric excess of the product (%), (△) enantiomeric excess of the substrate (%).

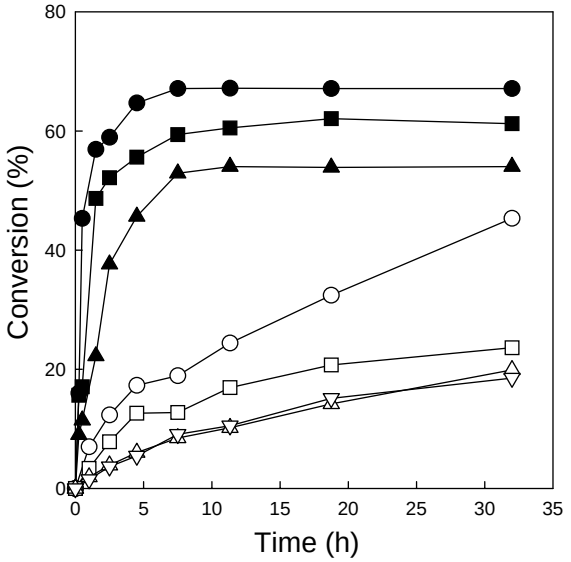

Fig. 5. Influence of alcohol chain length of the substrate on the hydrolysis of ketoprofen esters. Reaction conditions: 4.9 mmol substrate, 0.3 g Novozym 435, 50 °C. (●) ketoprofen methyl ester, (■) ketoprofen ethyl ester, (▲) ketoprofen propyl ester, (○) ketoprofen butyl ester, (□) ketoprofen hexyl ester, (△) ketoprofen octyl ester, (▽) ketoprofen decyl ester.

partition into the aqueous phase and reduce the final conversion.

As far as the influence of alcohol chain length on enantioselectivity is concerned, propyl and butyl esters of ketoprofen showed optimal values (Fig. 6). Enantioselectivity of Novozym 435 for these esters (about 8.0) was comparable to that observed for the esterification of ketoprofen with alcohols in optimal organic solvents (about 7–12) [12,13]. Furthermore, the productivities, defined as the rate of ester hydrolysis per enzyme weight (μmol h^{-1} g^{-1}), obtained in this study were much higher than those reported previously for the Novozym 435-catalyzed kinetic resolution of racemic ketoprofen. For example, in the case of the enzymatic hydrolysis using ketoprofen propyl ester, the productivity of the solvent-free two-phase system (2.170 μmol h^{-1} g^{-1}) was about 10–100 times higher than that of ketoprofen esterification with *n*-dodecanol [13] and ethanol [12] in organic solvents. Therefore, enzymatic hydrolysis in a solvent-free two-phase system can perform kinetic resolution of ketoprofen ester efficiently without solvents.

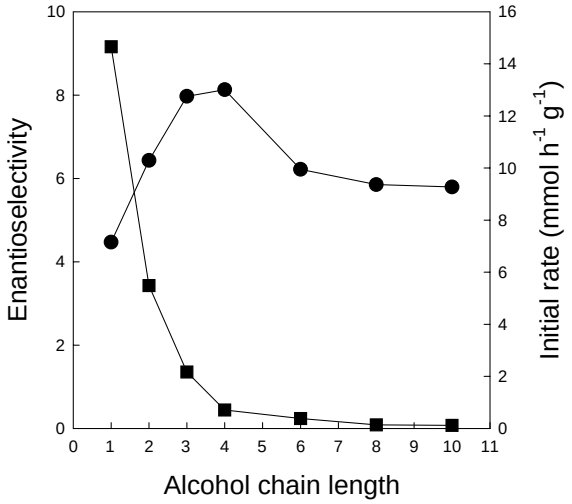

Fig. 6. Effect of the chain length of the alcohol moiety on enantioselectivity (●) and on initial reaction rate (■).

3.5. Reusability of the biocatalyst

Finally, the reusability of the biocatalyst was investigated at three different temperatures (50, 60, and 70 °C) using ketoprofen propyl ester as a substrate (Fig. 7). To investigate the reusability of Novozym

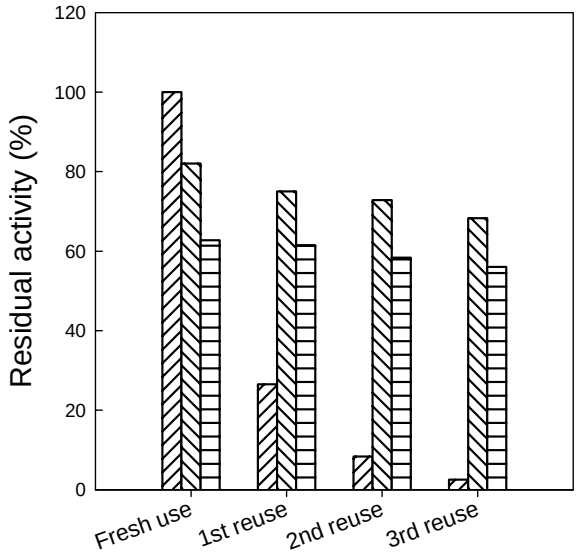

Fig. 7. Reusability of the biocatalyst (Novozym 435). (▨) 70 °C, (▧) 60 °C, (▤) 50 °C. The activity of fresh enzyme preparations at 70 °C was designated as 100% relative activity.

435, the enzymatic hydrolysis reaction was carried out in a solvent-free two-phase system using a vial containing 4.9 mmol ketoprofen propyl ester, 25 ml 0.1 M phosphate buffer (pH 8.0), and 0.3 g enzyme powder. To extract the unreacted substrate from the enzyme particles, *n*-hexane (10 ml) was added to the vial after the reaction and then the mixture was shaken rigorously. The enzyme particles were recovered by filtration and were dried in vacuo at room temperature for 6 h. The next cycle of enzyme reaction was conducted with fresh reagents and the enzymes recovered.

The results (Fig. 7) show that the reusability of Novozym 435 decreased with increasing reaction temperatures due to thermal inactivation of enzymes during the reaction. At 70 °C, the residual activity of Novozym 435 was lowered to 30% of initial activity after the first reuse and most of enzyme activity was lost after the third cycle. On the other hand, Novozym 435 retained 85 and 90% of its activity at 60 and 50 °C, respectively, after the third reuse. In view of this result, it appears that the operation of a reaction system at about 60 °C is appropriate for the production of optically pure ketoprofen in a solvent-free two-phase system using Novozym 435 as a catalyst.

3.6. Concluding remarks

In this study, we developed a solvent-free two-phase system in order to hydrolyze ketoprofen esters enantioselectively. The stereopreference of Novozym 435 allowed the production of (*S*)-ketoprofen ester as an unreacted substrate and (*R*)-ketoprofen as a by-product by the enzymatic reaction. Enantiomerically pure (*S*)-ketoprofen can be obtained easily by the chemical hydrolysis of (*S*)-ketoprofen ester. The solvent-free two-phase system, using ketoprofen butyl ester, produced the best results and this process can be easily scaled up for the large-scale production of enantiopure ketoprofen. Moreover, this novel solvent-free two-phase system may make it possible to produce other chiral drugs at high substrate concentrations. As the solvent-free system does not use harmful organic solvents, this process lends itself to environmentally friendly production.

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