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Enhancing effect of Tween-80 on lipase performance in enantioselective hydrolysis of ketoprofen ester

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Abstract

An additive method has been developed to obtain nearly optically pure (*S*)-ketoprofen [(*S*)-2-(3-benzoyphenyl)propionic acid, > 96% enantiomeric excess, ee] by using lipase OF (from *Candida rugosa*) for the first time. The effect of surfactants (selected as additives) on the enantioselective hydrolysis of 2-chloroethyl ester of ketoprofen by the crude and the purified *C. rugosa* lipase (lipase OF) was investigated. Except for Tween-60, Tween-80 and nonyl phenol polyethyleneoxy ether (OP-10), most of the other surfactants tested had inhibitory influence on the lipase. As for the enantioselectivity, only Tween-80 displayed a positive effect. Moreover, the concentration of Tween-80 was found to be a sensitive factor affecting the activity and the enantioselectivity, when either the crude or the purified enzyme was used. Upon addition of the two emulsifiers at their optimal concentrations, i.e., 2% (w/v) Tween-80 and 3% (w/v) OP-10, the crude enzyme activity was greatly enhanced up to 13 and 15 times, respectively. On the other hand, the enantiomeric ratio (*E* value) increased from 1.2 to 6.7 for the crude lipase and from 8 to > 100 for the purified lipase in the presence of 8% or 2% (w/v) Tween-80.

Keywords: *Candida rugosa* lipase; Enantioselectivity; Activity; Ketoprofen; Tween-80

1. Introduction

Ketoprofen (2-(3-benzoylphenyl)propionic acid), belonging to the family of 2-arylpropionic acids (profens), is one kind of the nonsteroidal anti-inflammatory drugs. Its (*S*)-enantiomer has the therapeutic activity of reducing inflammation and relieving pains, while the (*R*)-enantiomer can be used as a toothpaste additive to prevent periodontal disease [1]. Various efforts have therefore been made to obtain optically pure ketoprofen.

The preparation of optically pure ketoprofen could be accomplished by enzymatic resolution of its corresponding racemic mixture [2–6]. Of the lipases from different organisms, the one

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from *Candida rugosa* has a preference for the (*S*)-enantiomer and has been widely used in the biocatalytic resolution of 2-arylpropionic acids [7–9]. However, the enantioselectivity (or enantiomeric ratio, *E*) of these enzymes towards ketoprofen is far from satisfactory (usually $E < 5$) for synthetic use [6,8,9]. On the other hand, the rate of hydrolysis of water-insoluble esters is severely limited because of the incomplete contact between the enzyme and the droplets of substrate. The aim of the current work is to improve the enantioselectivity and activity of the *C. rugosa* lipase in the enzymatic hydrolysis of ketoprofen ester.

Among the methods reported [2–4,10–14] to increase the enantioselectivity of enzyme-mediated reactions, the additive approach is very attractive because of its simplicity for practical use, though only a few additives have so far been reported [11–15]. Furthermore, for the enzymatic hydrolysis of ketoprofen ester, achieving an improvement in enantioselectivity by additive method is to our knowledge without precedent in the literatures.

Surfactants are well known to be applied in lipase assay to increase the lipid–water interfacial area, which, in turn, enhance the observed rates of lipase-catalyzed reactions [16]. Since the reaction rate is strongly limited by the low solubility and the high viscosity of 2-chloroethyl ester, various surfactants were tested in this work as additives to the enzymatic reaction to enhance the productivity. Further study showed surprisingly that Tween-80 could also significantly enhance the lipase enantioselectivity, which was crucial for the enzymatic resolution. Based on this new discovery, an efficient additive method for improving the enzyme selectivity was thus developed and nearly optically pure (*S*)-ketoprofen (96.4% enantiomeric excess, ee) was successfully obtained.

It should be pointed out that lipase OF (from *C. rugosa*) was selected in this work to hydrolyze the ketoprofen ester exactly because it is economically competitive and has higher activity compared to the other lipases from *C. rugosa* (e.g., lipase MY and the Sigma lipase type VII), although it usually has a very low enantioselectivity.

2. Experimental

2.1. Materials

Lipase OF and lipase MY (both from *C. rugosa*) were products of Meito Sangyo, Japan. Another *C. rugosa* lipase (type VII) was from Sigma, USA. Ketoprofen was provided by Xinan Synthetic Pharmaceutical Factory, Chongqing, China. SP-Sephadex C-50 ion exchanger was from Pharmacia Biotech, Sweden. The racemic 2-chloroethyl ester of ketoprofen was prepared using the method described by Moreno and Sinisterra [9]. Nonyl phenol polyethyleneoxy ether (emulsifier OP-10) was bought from Tianjin Tiantai Reagent, China. Tween-80 was from Shanghai Dazhong Pharmaceutical Factory, China. All other chemicals were also obtained commercially and of analytical grade.

2.2. Purification of enzyme

Crude lipase OF was purified according to the procedure described previously with a little modification [4]. Five grams of crude enzyme powder was suspended in 200 ml of buffer (7 mM citrate–15 mM Na_2HPO_4, pH 3.3). The suspension was stirred for 20 min at 4°C followed by centrifugation ($13{,}700 \times g$, 20 min, 4°C). The supernatant was then dialyzed overnight against the same buffer (pH 3.3) and was centrifuged again. The clear solution was loaded on a SP-Sephadex C-50 column ($\varnothing 4 \times 30$ cm) and washed orderly with the citrate-phosphate buffers of pH 3.3, 4.7 and 6.8, with which the active fraction eluted was designated as L1, L2, L3, respectively. The protein concentration was determined using Bradford method with bovine serum albumin as a standard [17].

2.3. Enzymatic hydrolysis of ketoprofen ester

A typical example is described as follows: to 10 ml of 50 mM sodium phosphate buffer (pH 7.0) was added 100 mg of 2-chloroethyl ester of ketoprofen (32 mM), 20 mg of lipase OF/5 mg of L2/50 mg of L3. A surfactant was added to the system when required. The mixture was incubated at 30°C in a rotary shaker at 160 rpm. Samples (0.1 ml) were periodically withdrawn from the reaction mixture and acidified to pH 2–3 by the addition of 0.1 M HCl solution. Then, the acid and ester were extracted into 1 ml of ethyl acetate and subjected to HPLC analysis.

2.4. Stability of enzyme against surfactants

Several samples of lipase OF or L2 in 50 mM sodium phosphate buffer (pH 7.0) were incubated at 30°C in the presence of surfactants with different concentrations. At regular intervals, 2-chloroethyl ester of ketoprofen was added to one of the incubated enzyme samples, and the hydrolysis was carried out to assay the residual activity as described above.

2.5. Analysis

The conversion ratio of reaction was determined by HPLC (Waters 510) using a silica gel C-18 column. The mobile phase was composed of methanol and water (85:15, v/v) at 0.8 ml/min. UV detector (at 254 nm) was employed for quantification. The activity of enzyme was expressed by the initial reaction rate (0–5% hydrolysis).

The enantiomeric excess (ee) of the acid and the ester was determined using a chiral column (25 cm, Chiralcel OJ, Daicel, Japan) with hexane/2-propanol/acetic acid (90:10:0.05, v/v) at 1.0 ml/min as mobile phase. The enantiomeric ratio, E, was calculated by the equation [18] $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$, where c represents the conversion ratio and ee_p represents the enantiomeric excess of the product.

3. Results and discussion

3.1. Effect of surfactants on catalytic activity of the lipase

Three kinds of surfactants (non-ionic, cationic and anionic) were screened for an efficient emulsifier of the hydrophobic substrate. The effect of the selected emulsifiers on the rate of hydrolysis was systematically investigated (Table 1). Most of the non-ionic surfactants such as Tween-60, Tween-80 and emulsifier OP-10 stimulated the lipase activity. Under the same conditions, the conversion ratios of ketoprofen ester in the presence of 5% (w/v) Tween-80 or OP-10 achieved 46% and 43%, respectively, compared with 8.7% in the absence of surfactants. As to Span-60 and dodecyl phosphate, the conversion ratios at 10 h were about the same as that of the reaction without any surfactants. In the case of the cationic and anionic surfactants, inhibitory effect on the lipase was observed. For example, the conversion ratio decreased from 8.7% to 2.2% when AOT was added.

Table 1
Effect of surfactants on the crude lipase OF catalyzed reaction[a]

Surfactant		Conversion (%)	ee_p (%)	E
None		8.7	8.3	1.2
Non-ionic	Tween-80	46	60	6.7
	Tween-60	24	6.4	1.2
	Dodecyl phosphate	8.0	3.2	1.1
	OP-10[b]	43	4.5	1.1
	Span-60	9.2	3.0	1.1
Anionic	AOT[c]	2.2	26	1.7
	Sodium dodecyl sulfate	5.0	2.0	1.1
Cationic	BODMAC[d]	4.9	6.0	1.1
	Benzethonium chloride	3.9	68	5.4

[a] Ten milliliters sodium phosphate buffer (pH 7.0), 100 mg 2-chloroethyl ester of ketoprofen (32 mM), 20 mg lipase OF and 5% (w/v) surfactant, incubated at 30°C and 160 rpm for 10 h.
[b] Nonyl phenol polyethyleneoxy ether.
[c] Bis (2-ethylhexyl) sodium sulfosuccinate.
[d] Bisoctadecyl dimethyl ammonium chloride.

Our results agree with the previous reports [19,20] that the enhancement of lipase activity strongly depends on the type of surfactants. The emulsification of the insoluble ketoprofen ester by the surfactants leads to the increased interfacial oil–water area, which is helpful for the improvement of lipase activity, as shown in Table 1. Even when the ester was pre-dispersed by magnetic stirring before the reaction, the observed rate could increase up to threefold (data not shown). On the other hand, the influence of surfactants was also related to their interaction with lipase at the oil–water interface [19]. In our experiments, the increased activity of the enzyme was not observed despite the fact that the substrate could be also emulsified by other surfactants except for Tween-60, Tween-80 and OP-10. This suggests that the nature of surfactants have great influence on the lipase. The inhibitory effect of ionic surfactants may be attributed to ionic interactions between the surfactant and the lipase, which induce an unfolding and denaturing of the enzyme [21]. For the non-ionic surfactants, the lipase was activated to more or less extent because there were only hydrogen-bonding and hydrophobic interactions with the lipase.

In a word, Tween-80 and OP-10 were suitable surfactants for the enhancement of hydrolysis rate of ketoprofen ester.

3.2. Effect of surfactants on lipase enantioselectivity

Our results showed that the crude lipase OF displayed only a very low enantioselectivity ($E = 1.2$) (Table 1), which is the possible reason that it has seldom been employed to resolve ketoprofen in previous literature. The effect of surfactants on the enantioselectivity of lipase-catalyzed hydrolysis of 2-chloroethyl ester of ketoprofen was also presented in Table 1. Except for benzethonium chloride and Tween-80, most of the other surfactants employed made the ee value decrease to a greater or less extent. Benzethonium chloride appeared to have made E increase from 1.2 to 5.4; however, it had strong inhibitory influence on the activity of the lipase, which makes it practically worthless. It is interesting that Tween-80 markedly enhanced the enantioselectivity of this reaction, with ee of the product [(*S*)-ketoprofen] increasing from 8.3% to 60% and the corresponding E value from 1.2 to 6.7.

In addition, Tween-80 was observed to be also effective in enhancing the E values of lipase MY and lipase type VII from Sigma in the enantioselective hydrolysis of the same ketoprofen ester by factors of 3 and 5, respectively (data not shown).

It has been proved that the commercial preparation of *C. rugosa* lipase (CRL) contains at least two different active components [22]. In our case, the crude lipase OF was fractionated into three components-L1, L2 and L3 by a slightly modified method of Cobbs et al. [4]. The total yields of protein recovery and activity recovery were 47.9% and 58.6%, respectively. The major protein peak (L2) contained 86% of the total activity recovered, and its purification factor was 3.4-fold.

Through the purification, the increase of ee value of the product was observed, which confirms the previous conclusion [2,4] that the purification can increase the enantioselectivity of the lipase. Nevertheless, when the purified component-L2 was used, the ee value achieved only 76%. Tween-80 was thus tested in order to improve further the enantioselectivity of the purified lipase. The results in Table 2 reveal the crucial role of Tween-80 in the enhancement of enantioselectivity of L2 and L3. In the presence of 5% Tween-80, the enantioselectivity of both L2 and L3 was significantly improved, resulting in the increase of the E values from 8 to > 100 for L2 and from 2 to 11 for L3.

3.3. Effect of surfactant concentration on the lipase performance

In a process of enzymatic resolution with a given surfactant as an additive, its concentration

Table 2
Effect of Tween-80 on two purified components (L2, L3) from lipase OF

Enzyme	Tween-80 (%, w/v)	Reaction time (days)	Conversion (%)	ee_p (%)	E
L2	0	1	13	76	8
	5	1	51	95	>100
L3	0	6	13	29	2
	5	4	45	72	11

The reactions were carried out by shaking at 160 rpm and 30°C the mixture of 10 ml phosphate buffer (pH 7.0), 100 mg ketoprofen ester (32 mM), 5 mg L2 or 50 mg L3, and when required, 5% (w/v) Tween-80. The total activities of 5 mg L2 or 50 mg L3 were about the same (1250 U, using PVA-emulsified olive oil as the substrate).

is another important parameter worthy of careful optimization, since it may also have deep influences on enzyme activity, enantioselectivity, and stability, among which the enantioselectivity seems to be the most crucial factor for the resolution. As for the issue of enzyme stability, it might be solved by various immobilization techniques.

Fig. 1 shows the different patterns of the dependence of the lipase activity on Tween-80 and OP-10 concentrations. The activity of lipase OF increased with the increase of Tween-80 concentration, but it was leveled off when the concentration exceeded 1%. As to OP-10, a concentration up to 3% could still improve the lipase activity, but further increase of concentration thereafter would make the lipase activity decrease. Therefore, the optimal concentrations

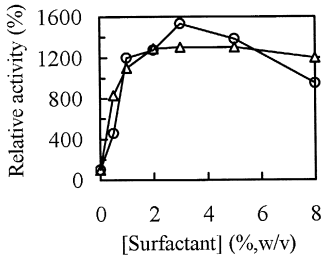

Fig. 1. Effect of surfactant concentration on the activity of crude lipase OF. (○) OP-10; (△) Tween-80. Reaction conditions were the same as in Table 1 except for 1 h. The enzyme activity with no surfactant (0.14 μmol mg^{-1} h^{-1}) was expressed as 100%.

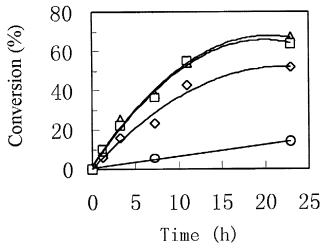

Fig. 2. Time courses of ketoprofen ester hydrolysis by L2 with various Tween-80 concentrations (w/v): (○) 0%; (◇) 0.3%; (□) 2%; (△) 8%. Reaction conditions were the same as in Table 2. L2 was an enzyme purified from lipase OF (see Section 2.1).

of Tween-80 and OP-10 were 2% (w/v) and 3% (w/v) in terms of the initial rate, where the activities of the crude lipase could be enhanced up to 13- and 15-fold, respectively. With respect to the purified enzymes from lipase OF in the presence of Tween-80, a similar effect was observed. The activities of L2 (Fig. 2) and L3 (data not shown) were about six times higher in the presence of 0.3% (w/v) Tween-80 than those without the surfactant. As shown in Fig. 2, it took only 10 h to obtain a conversion of 50% in the presence of 2% Tween-80, whereas the reaction proceeded to only 14% after 24 h in the absence of Tween-80. When the concentration was higher than 2%, the activity could not be further enhanced. Therefore, excessive surfactant was useless for the activity of the purified enzyme as well as the crude one.

Similar to the activity, the enantioselectivity of the crude and purified enzymes also depends on the concentration of Tween-80 (Fig. 3). Within the range of 0–8% (w/v) Tween-80, the

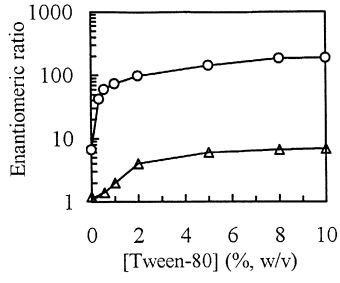

Fig. 3. Effect of Tween-80 concentration on the E value of crude lipase (△) and L2 (○). Reaction conditions were the same as in Table 2 except for 20 mg of lipase OF and 5 mg of L2.

E value of the crude lipase gradually increased from 1.2 to 6.7, while above 8%, no further enhancement was observed. For L2, which contained most of the lipase activity recovered, the E value increased with the increase of Tween-80 concentration, and attained 100 when the concentration was 2%. Up to 10%, the E value was still maintained at a high level (> 100). Hence, in the presence of 2% Tween-80, a product of (S)-ketoprofen could be prepared, with a pretty high optical purity (96.4% ee), through the L2-catalyzed hydrolysis of ketoprofen ester at 49.5% conversion. As a consequence, simply by the addition of Tween-80, an efficient method was developed, which could lead to a dramatic improvement of the enzyme enantioselectivity in the hydrolysis of racemic ketoprofen ester.

3.4. Stability of lipase in the presence of surfactants

Fig. 4 shows the inactivation profiles of lipase OF in the presence of 2% (w/v) Tween-80 or 3% (w/v) OP-10, compared with that without any surfactants. It was obvious that both of the surfactants diminished the stability to a certain content. Indeed at 30°C and pH 7.0, the lipase lost only 50% activity in the absence of surfactants after 30 h, while only 25% activity was left in the presence of surfactants. However, it should be pointed out that the curves level off when incubation time attains 30 h.

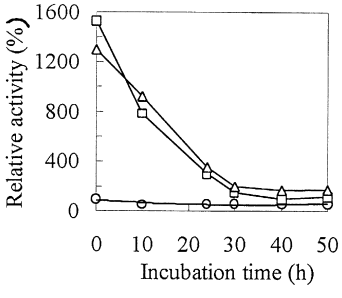

Fig. 4. Stability of lipase OF in buffer solution at 30°C and pH 7.0 in the presence of surfactants. (○) The control without surfactants; (□) 3% OP-10; (△) 2% Tween-80. The activity at 0 h in the absence of surfactants (0.14 μmol mg^{-1} h^{-1}) was expressed as 100%.

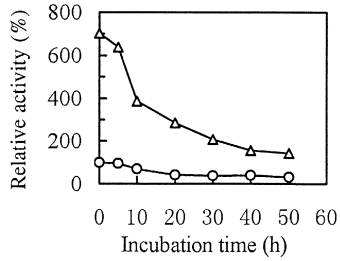

Fig. 5. Stability of L2 in buffer solution at 30°C and pH 7.0 in the presence of Tween-80. (○) The control without surfactants; (△) 2% Tween-80.

Moreover, the activity of the lipase in the presence of surfactants was still two to three times higher than those without surfactant after 50 h.

Since the successful resolution was achieved with L2, its stability was also examined. As illustrated in Fig. 5, the negative effect of Tween-80 was observed on L2 as well as the crude one. Being incubated at 30°C and pH 7.0 for 50 h, L2 lost 70% and 80% activity without or with the addition of Tween-80, respectively. Therefore, it is of great importance to enhance the stability of the enzyme in order to achieve a practical application of this additive method. Fortunately, our preliminary experiments have shown that the stability of enzyme could attain a satisfactory level through immobilization.

4. Conclusion

Among various approaches to increase the enantioselectivity of enzymes, the additive method is the simplest. However, it is not always successful to find a suitable additive. The present study demonstrates that Tween-80 has the potential to greatly enhance the reaction rate and the enantioselectivity in the lipase-catalyzed hydrolysis of 2-chloroethyl ester of ketoprofen.

In this system, Tween-80 seems to play a three-sided role. First, it serves as an emulsifier to give a stable substrate emulsion, which provides a large interfacial area. Second, the nature of Tween-80 enables it to be an activator of the

lipase. Finally, the most important is its ability of enhancing the enantioselectivity. In a word, our approach can be considered as a new and facile method for the efficient resolution of racemic ketoprofen by using relatively inexpensive but highly active lipase OF as biocatalyst.

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