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Catalytic performance of a highly enantioselective (*R*)-ester hydrolase from a new isolate *Acinetobacter* sp. CGMCC 0789

Jing-Hua Qian, Jian-He Xu*

Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, P.O. Box 283, 130 Meilong Road, Shanghai 200237, PR China

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Abstract

A highly enantioselective (*R*)-ester hydrolase was partially purified from a newly isolated bacterium, *Acinetobacter* sp. CGMCC 0789, whose resting cells exhibited a highly enantioselective activity toward the acetate of (4*R*)-hydroxy-3-methyl-2-(2-propynyl)- cyclopent-2-enone (*R*-HMPC). The optimum pH and temperature of the partially purified enzyme were 8.0 and 60 °C, respectively. The enantioselectivity of the crude enzyme was increased by 1.2-fold from 16 to 20 when the reaction temperature was raised from 30 to 60 °C. The activity of the crude enzyme was enhanced by 4.1-fold and the enantioselectivity (*E*-value) was markedly enhanced by 4.3-fold from 16 to 68 upon addition of a cationic detergent, benzethonium chloride [(diisobutyl phenoxyethoxyethyl) dimethyl benzylammoniom chloride]. The hydrolysis of 52 mM (*R*,*S*)-HMPC acetate to (*R*)-HMPC was completed within 8 h, with optical purity of 91.4% ee_p and conversion of 49%.

Keywords: *Acinetobacter* sp.; Esterase; Enantioselectivity; Allethrin; Enzymatic resolution

1. Introduction

Allethrin, belonging to the family of synthetic pyrethroids that are a group of ester compounds, has excellent insecticidal activities and low toxicity to mammals. It has very fast knock-down and lethal effects against household insect pests. 4-Hydroxy-3-methyl-2-(2-propynyl)-cyclopent-2-enone (HMPC) is the alcohol moiety of allethrin and studies on the relationship between the stereochemistry and insecticidal activity have revealed that the (*S*)-form of the HMPC is the active stereoisomer in allethrin [1]. The insecticidally active (*S*)-HMPC can be prepared from the (*R*,*S*)-HMPC acetate by a combination of lipase-catalyzed hydrolysis and chemical transformation with inversion of chiral configuration. Mitsuda et al. (1998) have described this process [2]—after the enzymatic reaction which enantioselectively hydrolyzes (*R*)-HMPC acetate (Scheme 1), the liberated (*R*)-HMPC could be sulphonated with an organic sulphonyl halide. The alkylsulphonate of (*R*)-HMPC obtained was easily hydrolyzed with a weakly alkaline solution to give chirality-inverted (*S*)-HMPC. The unhydrolyzed (*S*)-HMPC acetate was inert during sulphonation reaction and retained its (*S*)-configuration during the chemical hydrolysis with a weakly alkaline solution.

Mitsuda et al. (1988) reported a highly enantioselective hydrolysis using a commercially available but expensive *Arthrobacter* lipase preparation. With *Arthrobacter* lipase, they obtained the optically pure (*R*)-HMPC at 50% hydrolysis in a two-liquid phase reaction system of water and the insoluble substrate. The hydrolysis proceeded even at a substrate concentration of 80% (w/v). They also tried to isolate microorganisms, but the highest ee_p was 90.2% at 11.7% hydrolysis in the case of 0.5% (w/v) substrate concentration with *E*-value of 22 [2].

Our laboratory has isolated a strain with the name of *Acinetabacter* sp. CGMCC 0789. It can hydrolyze the (*R*,*S*)-HMPC acetate with 92.3% ee_p at 48.8% hydrolysis in the case of 1% (w/v) substrate concentration with *E*-value of 73 [3].

Variation of the temperature to enhance the enantioselectivity has been known for years. In many cases, the selectivity decreases with increasing temperature [4]. However, this is not always the case. In our study, the selectivity of the esterase increased with the increase of temperature.

In the aqueous reaction system with the insoluble (*R*,*S*)-HMPC acetate, the hydrolysis rate of substrate imposed a

* Corresponding author. Fax: +86-21-6425-2250.
E-mail address: jianhexu@ecust.edu.cn (J.-H. Xu).

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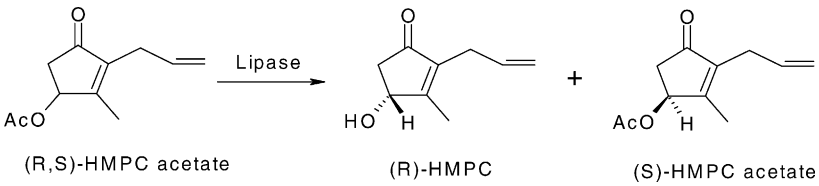

Scheme 1. The kinetic resolution of (*R*,*S*)-HMPC acetate catalyzed by a lipase [2].

task upon practical use because of the incomplete contact between the enzyme and the droplets of water-insoluble substrate. To increase the activity and enantioselectivity of the esterase in the enzymatic hydrolysis of HMPC acetate, addition of a suitable surfactant is a very attractive method due to its easy handling and low cost for practical use. Similar approaches have also been reported by other researchers [5,6].

We here partially purified an esterase from a newly isolated *Acinetobacter* sp. CGMCC 0789 with high selectivity to (*R*)-HMPC acetate. And the enzyme was characterized for the development of a practical enzymatic process. Because the enzyme could efficiently hydrolyze (*R*)-HMPC acetate with a high conversion and enantioselectivity, we anticipate the enzyme to be potentially useful as a practical biocatalyst for the enzymatic resolution of (*R*,*S*)-HMPC acetate in a large scale.

2. Experimental

2.1. Chemicals

(*R*,*S*)-HMPC acetate was provided by Yangzhou Agrochemical Co., Jiangsu Province, China. Butyl-Toyopearl was purchased from Tosoh Corp, Tokyo, Japan. DEAE-cellulose was purchased from Pharmacia Biotech, Shanghai, China. The *p*-nitrophenyl esters with different length of acyl moieties were prepared using the method described by Wynn et al. [7]. Benzethonium chloride was purchased from Tokyo Kasei Kogyo Co. Ltd., Japan. All other chemicals were also obtained commercially and of analytical grade.

2.2. Microbial strain and culture conditions

Acinetobacter sp. CGMCC 0789 was used throughout this study. It was isolated from soil by Zhao et al. [3] and presently deposited at China General Microorganism Collection Center with an accession number of CGMCC 0789. The cells were cultured aerobically at 30 °C for 24 h in an optimized medium containing 1% sucrose, 0.5% peptone, 0.1% yeast extract, 0.02% KH_2PO_4, 0.01% NaCl, 0.02% $MgSO_4{\cdot}7H_2O$ (pH 7.0).

2.3. Partial purification of enzyme

Cells were harvested from 3.0 l broth of a 24 h culture in a 5 l jar fermenter, washed twice with physiological saline (0.85% NaCl). A crude extract was prepared by sonification for 20 min of cells (15 g, wet weight) suspended in 60 ml of buffer A (50 mM Tris–HCl, pH 7.5, containing 1 mM cysteine and 1 mM EDTA). All the purification steps were performed at 0–4 °C and Tris–HCl buffer was used as a standard buffer. The disrupted cells were centrifuged at 270,000 × *g* for 30 min. The supernatant was collected and fractionated with ammonium sulfate (70–90% saturation). The active precipitate was dialyzed overnight against buffer B (10 mM Tris–HCl, pH 7.5, containing 1 mM cysteine and 1 mM EDTA) before applied onto a DEAE-cellulose column (Φ3 × 15 cm) equilibrated with buffer B. The column was washed with 120 ml buffer B, and then eluted with 0–1 M linear gradient of NaCl in buffer B (total volume, 400 ml; flow rate, 0.8 ml/min; fraction volume, 4.0 ml). Active fractions were pooled and ammonium sulfate was added up to a concentration of 1.0 M. The solution was then applied onto a Butyl-Toyopearl column (Φ2.4 × 6 cm) which had been equilibrated with buffer B containing 1.0 M ammonium sulfate. The enzyme was eluted with a linear gradient of 1.0–0 M ammonium sulfate in buffer B.

2.4. Enzyme and protein assay

The activity of the esterase was usually measured using the HMPC acetate as a substrate. A typical example is described as follows: the enzyme solution (0.2 ml) was diluted with 0.8 ml of 100 mM potassium phosphate buffer (KPB, pH 7.0). The reaction was started by the addition of (*R*,*S*)-HMPC acetate to a final concentration of 52 mM. A surfactant was added to the system when required. The mixture was incubated at 30 °C in a rotatory shaker at 160 rpm. At the end of reaction, both the alcohol produced and the residual ester were extracted into 1 ml of ethyl acetate and subjected to GC analysis.

For a rapid assay during purification of the esterase, the activity of the esterase was measured spectrophotometrically using *p*-nitrophenyl acetate (PNPA) as a substrate. The enzymatic hydrolysis of the substrate was monitored by the amount of *p*-nitrophenol released at 25 °C. The standard assay mixture contained 1 mM of PNPA dispersed in 3 ml of 100 mM potassium phosphate buffer (KPB, pH 7.0) with 30 μl of DMSO. After preincubation at 25 °C for 3 min, the reaction was started by addition of 0.1 ml enzyme solution and the change in absorbance at 405 nm was recorded with a spectrophotometer. The molecular extinction coefficient of *p*-nitrophenol under the assay conditions above was 9873 $M^{-1}\,cm^{-1}$. One unit of the esterase activity was

defined as the amount of the enzyme producing 1 μmol of *p*-nitrophenol in 1 min.

The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard [8]. During the chromatographic purification, the protein content of fractions was routinely estimated by measuring the UV absorbance at 280 nm.

Sodium dodecyl sulfate-polyacryamide gel eletrophoresis (SDS-PAGE) was performed according to method of Laemmli [9], using 12.5% poly-acrylamide gel containing 0.1% SDS.

2.5. Effects of pH, temperature and metal ions

The optimum pH of the esterase activity was determined using three different buffers: Na_2CO_3–$NaHCO_3$ (pH 9–10), $NaHPO_4$–KH_2PO_4 (pH 5–7), and Tris–HCl (pH 7–9). The pH effect on stability was tested by preserving the enzyme preparation in buffers of various pHs for 1 h and the residual activity was assayed (at pH 8.0) using racemic HMPC acetate as substrate. The assay with PNPA as substrate is not suitable for studying the effect of pH on the hydrolytic activity because the absorbance of *p*-nitrophenol varies considerably with the pH of the solution [10].

The temperature effect on activity was examined by measuring the activities with PNPA as substrate at controlled temperatures in the range of 20–80 °C. The thermal stability of enzyme was determined by exposing the enzyme, before testing, to specified temperatures for 1 h. The enzyme solution was then cooled and the residual activity was assayed spectrophotometrically at 40 °C.

The effect of metal ions on enzyme activity was examined by measuring the enzyme activity with PNPA as substrate at 25 °C. Before substrate was added, the enzyme was preincubated with various metal ions (5 mM) for 5 min.

2.6. GC analysis, Eantiomeric ratio (E) and kinetic constants determination

The substrate and product concentrations were determined by GC using BETA DEX™ 120 column (column temperature, 160 °C; injector and detector temperature, 280 °C) to determine the degree of hydrolysis and enantiomeric excesses of substrate (ee_s) and product (ee_p). The retention times for (*S*)-HMPC acetate, (*R*)-HMPC acetate, (*S*)-HMPC, (*R*)-HMPC were 11.2, 11.6, 14.5, 14.9 min, respectively. Enantiomeric ratio (*E*) was calculated using the equation of [11]:

$$E = \ln\left[\frac{(1 - ee_s)/(1 + ee_s/ee_p)}{(1 + ee_s)/(1 + ee_s/ee_p)}\right]$$

Kinetic constants were determined by the Lineweaver–Burk method, using (*R*,*S*)-HMPC acetate as the substrate.

3. Results and discussion

3.1. Properties of partially purified esterase

The result of purification is summarized in Table 1. On the whole, the crude enzyme was partially purified up to about 50-fold higher specific activity through fractionation with ammonium sulfate (70–90% saturation) and column chromatography with DEAE-cellulose and Butyl-Toyopearl, affording an overall yield of 15% from the crude cell-free extract. Allowing for the yield of enzyme activity, partially purified enzyme after DEAE-cellulose chromatography was used in subsequent studies of enzyme properties. In the chromatography of Butyl-Toyopearl, the enzyme was eluted out at zero concentration of ammonium sulfate and the specific activity of the enzyme was greatly enhanced. It means that the esterase had a strong hydrophobic interaction with the matrix. We suppose that the esterase has a specific interaction with butyl on the matrix that may just act as an analog of the enzyme substrate.

After these steps, there were still two protein bands appearing on SDS-PAGE using silver staining (Fig. 1). The molecular weights of the two proteins are about 20 and 14 kD, respectively. The two bands still appeared after native-PAGE. So they are not the subunits of the esterase. According to literature [12], 14 kD is rarely reported for the molecular weight of esterase. So it is supposed that the molecular weight of the esterase is around 20 kD which is rather small among those reported so far.

Substrate specificity was analyzed by the ability to release of *p*-nitrophenol from various ester compounds (Table 2). As shown in Table 2, the enzyme showed the highest

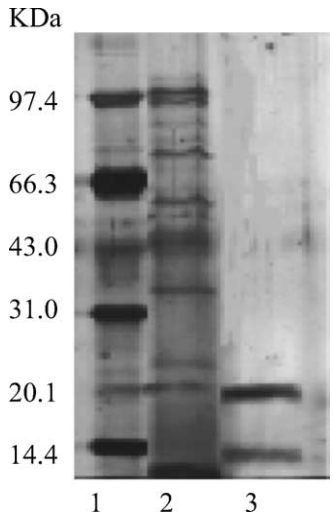

Fig. 1. SDS-PAGE of the partially purified esterase from *Acinetobacter* sp. CGMCC 0789. Lane 1, molecular mass standards (from top to bottom): phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa). Lane 2, an enzyme sample collected after DEAE-cellulose chromatography. Lane 3, an enzyme sample collected after Butyl-Toyopearl chromatography.

Table 1
Summary of esterase purification

Purification step	Total activity[a] (U)	Total protein (mg)	Sp. act.[a] (U/mg)	Purification factor (fold)	Yield (%)
Cell free extract	32.8	267	0.123	1	100
$(NH_4)_2SO_4$ (70–90%)	20.6	184	0.111	0.9	63
DEAE-cellulose	9.9	25.7	0.385	3.1	30
Butyl-Toyopearl	5.0	0.8	6.07	49	15

[a] Enzyme activity was determined using PNPA as a substrate, as described in Section 2.

activity toward *p*-nitrophenyl butyrate (PNPB) which is a typical substrate of an esterase. These experiments also showed that long chain esters such as *p*-nitrophenyl laurate were hydrolyzed with very low activity further supporting that the enzyme is a true carboxyl esterase and not a lipase.

Effects of pH and temperature (30–60 °C) on activities of the esterase were studied by measuring the activity of the partially purified enzyme after DEAE-cellulose chromatography in buffers at various temperatures and pHs (data not shown). The enzyme showed the maximum activity at 60 °C and retained about 60% of activity at 60 °C after incubation for 1 h. In addition, the enzyme activities were measured in the pH range of 5.0–9.0, and the optimum pH of the enzyme was observed at pH 8.0.

In order to study the effect of metal ions on the enzyme activity, the enzyme was incubated with various metal ions (5 mM) for 5 min before activity assay using *p*-nitrophenyl acetate (PNPA) as the substrate. As a result, $Fe^{3+}$and Cu^{2+} inhibited the activity of the enzyme obviously, while Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Pb^{2+}, Ag^{+} have a stimulating effect on the enzyme activity. Especially, Ca^{2+} has almost 150% enhancing effect on the activity of the enzyme.

3.2. Effects of temperature on the enzyme activity and enantioselectivity

From the results shown above, we found that high temperature has a positive effect on the activity of the enzyme. So hydrolysis of the HMPC acetate at different temperatures was compared between 60 and 30 °C under standard reaction conditions using the crude enzyme extract. It was found that the enzyme activity at 60 °C was enhanced by 2.0-fold and enantioselectivity was enhanced by 1.2-fold from 16 to 20, as compared to that at 30 °C (Fig. 2).

Table 2
Substrate specificity of the esterase from *Acinetobacter* sp. CGMCC 0789

Substrate	Specific activity[a] (U/mg)	Relative activity (%)
p-Nitrophenyl acetate	0.251	53
p-Nitrophenyl propionate	0.369	78
p-Nitrophenyl butyrate	0.474	100
p-Nitrophenyl caproate	0.352	74
p-Nitrophenyl caprate	0.297	63
p-Nitrophenyl laurate	0.082	17

[a] Enzyme activity was determined using the partially purified enzyme (after DEAE-cellulose chromatography) in the presence of corresponding substrate (1.0 mM) in 3 ml of 100 mM potassium phosphate buffer (KPB, pH 7.0) buffer.

3.3. Effects of surfactants on the enzyme activity and enantioselectivity

Three kinds of surfactants (non-ionic, cationic and anionic) were screened for an efficient emulsifier of the hydrophobic substrate using the crude enzyme extract. The effect of the selected emulsifiers on the hydrolysis rate was systematically investigated (Table 3). Cationic surfactants such as bis(octadecyl) ammonium chloride (BODMAC), cetyltrimethyl ammonium bromide (CTAB), benzethonium chloride stimulated the esterase activity. Under the same conditions the specific activity of enzyme using 0.25% (w/v) benzethonium chloride and BODMAC as additives were 0.130 and 0.128 U mg per protein, respectively, compared with 0.052 U mg per protein in the absence of surfactants. In the case of the anionic and non-ionic surfactants, no significant inhibiting or stimulating effect on specific activity or enantioselectivity was observed. Cationic surfactants (benzethorium chloride and CTAB) could also significantly enhance the enantioselectivity of the enzyme. *E*-values were 60 and 48 in the case of benzethorium chloride and CTAB, respectively, in contrast to 16 in absence of the surfactants. Benzethorium chloride had the most profound effect on the enzymatic reaction. It enhanced the specific activity by 2.5-folds and *E*-value by 3.7-folds.

In a process of enzymatic resolution with a given surfactant as additive, surfactant concentration is another

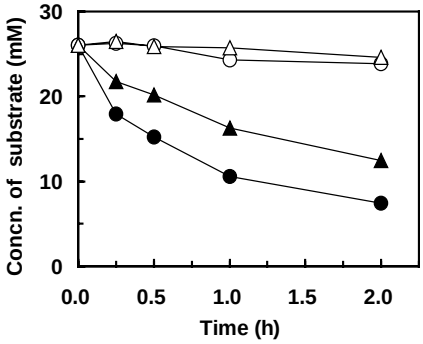

Fig. 2. Comparison of the enzymatic hydrolysis of (*R*,*S*)-HMPC acetate (52 mM) between 60 and 30 °C using the crude enzyme—variation in concentrations of each enantiomer of HMPC acetate with time. Symbols, (●) (*R*)-substrate (60 °C); (○) (*S*)-substrate (60 °C); (▲) (*R*)-substrate (30 °C); (△) (*S*)-substrate (30 °C).

Table 3
Effect of surfactants on the hydrolysis of (*R*,*S*)-HMPC acetate by the esterase from *Acinetobacter* sp. CGMCC 0789

Detergent[a]	Specific activity (U/mg)	Relative activity (%)	*E*-value[b]
No addition	0.052	100	16
Anionic			
SDS[c]	0.048	92	22
Deoxycholic acid Na-salt	0.055	106	14
Sodium dodecyl benzene sulfonate	0.069	133	19
AOT[d]	0.072	138	28
Non-ionic			
Tween-80	0.050	96	24
Span-60	0.054	104	20
Triton X-100	0.056	108	21
OP-10[e]	0.062	119	24
Tween-60	0.064	123	22
Cationic			
CTAB[f]	0.096	184	48
BODMAC[g]	0.128	246	40
Benzethonium chloride	0.130	250	60

[a] The concentration of the detergent is 0.25% (w/v).
[b] Calculated as $E = \ln[(1 - ee_s)/(1 + ee_s/ee_p)]/\ln[(1 + ee_s)/(1 + ee_s/ee_p)]$.
[c] Sodium dodecyl sulfate.
[d] Bis (2-ethylhexyl) sodium sulfosuccinate.
[e] Nonyl phenol polyethyleneoxy ether.
[f] Cetyltrimethyl ammonium bromide.
[g] Bis(octadecyl) ammonium chloride.

important parameter worthy of careful optimization, since it may also have deep influences on enzyme activity and enantioselectivity, among which the enantioselectivity seems to be the most crucial factor for the resolution. Benzethonium chloride was chosen because this detergent has the largest enhancing effect on the reaction rate and enantioselectivity.

As shown in Fig. 3, the maximum specific activity of the enzyme was obtained in the presence of 0.25% (w/v) surfactant, while the maximum *E*-value (68) was reached at 0.5% (w/v) surfactant. Since enantioselectivity is more important for our consideration, we chose 0.5% (w/v) as the optimum concentration for our further study.

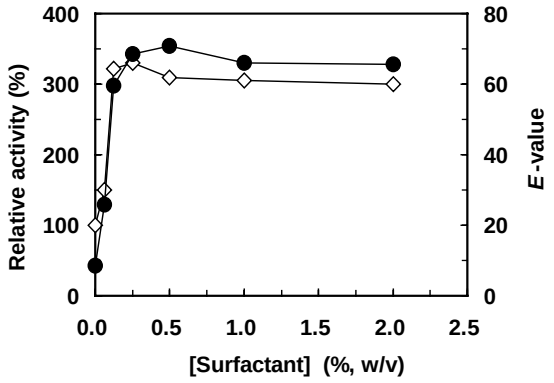

Fig. 3. Effect of benzethonium chloride concentration on the bioconversion rate and enantioselectivity. The enzyme activity with no surfactant was expressed as 100%. Symbols, (●) *E*-value; (◇) enzyme activity.

3.4. Kinetic resolution of (R,S)-HMPC acetate by crude enzyme extract from Acinetobacter sp. CGMCC 0789

The kinetics of biocatalytic resolution of racemic HMPC acetate was examined using crude enzyme extract of *Acinetobacter* sp. CGMCC 0789 as a biocatalyst at 30 °C, with benzethonium chloride as additive, in comparison with a control without any additive. The changes in concentrations of the substrate and the product with time are shown in Fig. 4.

After 8 h of reaction, using benzethonium chloride as additive, the ee_p and conversion were 91.4 and 49%, respectively, compared with 84.5 and 41% in absence of any additive. The specific activity was enhanced by 4.1-fold and the *E*-value was enhanced by 4.3-fold from 16 to 68.

As a consequence, simply by the addition of a surfactant, an efficient method was developed, which could lead to significant improvement of the enzyme enantioselectivity in the hydrolysis of racemic HMPC acetate.

There are also other reports about enhancement of enzyme activity and enantioselectivity using various surfactants [5,6,12–15]. Two reasons were presented to explain this phenomenon—the emulsification of the insoluble substrate by surfactants could lead to increased oil–water interfacial area, which is helpful for the improvement of esterase activity. In our experiments when increasing the speed of stirring, the reaction rate could also be increased to some extent.

We also tried to understand the surfactant activation mode of the esterase by using Lineweaver–Burk plots (data not shown). The K_m and V_{max} of the esterase were 32.5 mM and 0.65 μmol/min per mg protein in the presence of benzethonium chloride (0.5%, (w/v)), in contrast to 385 mM and 1.43 μmol/min mg per protein in absence of the surfactant. There is a marked (5-fold) increase in V_{max}/K_m value with

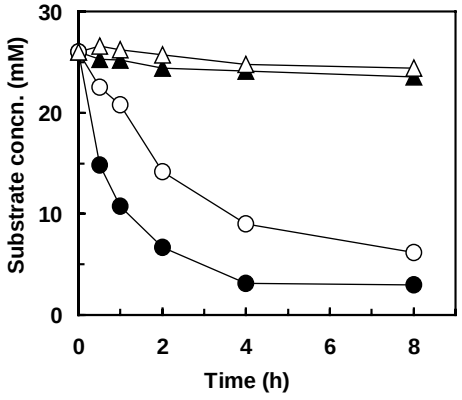

Fig. 4. Comparison of the enzymatic hydrolysis of (*R*,*S*)-HMPC acetate between reactions at 30 °C with and without benzethonium chloride—time-dependent changes in each enantiomer concentration of the racemic substrate (52 mM). The concentration of benzethonium chloride was 0.5% (w/v). Symbols, (●) (*R*)-substrate with surfactant; (▲) (*S*)-substrate with surfactant; (○) (*R*)-substrate without surfactant; (△) (*S*)-substrate without surfactant.

benzethonium chloride as additive compared with the case of no additive, indicating an increase in affinity of the substrate with the enzyme. So enhanced activity and enantioselectivity may be also due to small changes in 3-D structure of esterase. In the case of lipase, the detergents could also activate the enzyme by inducing opening of a surface loop ("lid") residing directly over the enzyme active site [13]. There was also a report on a cationic surfactant (CTAB) that influences the activity of the potato acid phosphatase by changing the conformation of the enzyme [12].

4. Conclusions

We have described a new, partially purified, esterase with high selectivity toward the ester of (*R*)-HMPC. The enantioselectivity of the enzyme could be increased when the reaction temperature was increased. Additionally, the enzyme activity and enantioselectivity were markedly enhanced by 4.1- and 4.3-fold, respectively, in the presence of a cationic surfactant, benzethonium chloride. The new enzyme, with high activity and enantioselectivity, from *Acinetobacter* sp. CGMCC 0789, might be effectively used as a new biocatalyst for the synthesis of optically pure (*S*)-HMPC combined with a chemical inversion.

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