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Screening, production and properties of a stereospecific esterase from *Pseudomonas* sp. S34 with high selectivity to (*S*)-ketoprofen ethyl ester

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Abstract

To isolate novel strains expressing an esterase that hydrolyzed the *rac*-ketoprofen ethyl ester to (*S*)-ketoprofen in the stereospecific manner, we screened broad ecological niches and soil samples in which the activity was expected to be found. Thousands of microbial strains were tested to determine their ester-hydrolyzing activity by using an agar plate containing insoluble tributyrin as an indicative substrate, and then further screened by activity on the (*R*,*S*)-ketoprofen ethyl ester. Twenty-eight strains were screened primarily and compared with respect to the potential to ketoprofen ethyl ester-hydrolyzing activity in terms of conversion yield and chiral specificity. Consequently, a strain S34 was isolated as a best producer and finally identified as a *Pseudomonas* sp. S34. We first formulated the optimal medium for the high level production of the enzyme, and as a preliminary experiment for enzymatic resolution, we characterized the fractionated enzyme. The enzyme with ketoprofen ethyl ester-hydrolyzing activity to (*S*)-ketoprofen showed a high degree of enantioselectivity (>94%) and was mainly found in cell extracts, whereas no distinct activity was detected in culture broth. The optimum pH and temperature of the enzyme were 9.5 and 35 °C, respectively. The activity of the enzyme was markedly increased (four-fold) by addition of a non-ionic detergent Triton X-100 and, resultantly, a high activity toward ketoprofen ethyl ester (52 U/mg) was found. The small-scale conversion of (*R*, *S*)-ketoprofen ethyl ester to (*S*)-ketoprofen using the partially purified enzymes was completed in 28 h, with optical purity of 99% and yield of 47%. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Screening; *Pseudomonas*; Stereospecificity; Esterase; (*S*)-Ketoprofen

1. Introduction

A commercially available anti-inflammatory drugs (NSAIDs) are known to be a class of 2-arylpropionic acids, such as ibuprofen [(*R*, *S*)-2-(4-isobutylphenyl) propionic acid], naproxen [(+)-(*S*)-2-(6-methoxy 2-naphtyl) propionic acid] and ketoprofen [(*R*,*S*)-2-(3-benzoylphenyl) propionic acid], which are non-steroidal and thus has been gained a lot of attention [1,2]. All of them contain a stereogenic centers in α-position of the carboxyl group. The pharmacological activity of 2-arylpropionic acids is mainly due to the (*S*)-enantiomer while its (*R*)-counterpart is biologically inactive or has a negative effect [3]. Therefore, considerable effort has been devoted to the synthesis of optically active (*S*)-2-arylpropionic acids.

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The chiral specific production of optically pure ketoprofen had been conducted by enzymatic resolution of its corresponding racemic mixtures [4–6]. Among the lipases and/or esterases reported from various sources, an enzyme from *Candida rugosa* has been known to have a relatively high activity to the (*S*)-enantiomer and therefore widely employed for the stereospecific resolution of (*R*, *S*)-ketoprofen ethyl ester to optically pure ketoprofen [7,8]. However, the enantioselectivity of the enzyme toward an arylpropionic acid, (*S*)-ketoprofen, is relatively low when the enzyme employed in general condition [7]. In addition, another problem, the rate of hydrolysis of water-insoluble (*R*, *S*)-ketoprofen ethyl ester, imposed a task upon practical use because of the incomplete contact between the enzyme and the water-insoluble droplets of substrate. To increase the rate of hydrolysis of water-insoluble ester in enzyme reaction, the addition of various emulsifier (surfactants) is very attractive due to its easier to handle and low cost for practical use [5]. Typical approaches for the surfactant additions are well known to the lipase process to increase the solubility of the water-insoluble lipid or to disperse the substrate, resulting the enhancement of enzyme activity and/or enantioselectivity in the lipase-catalyzed enzyme resolution of various esters. There was a similar problem reported in the esterase-mediated kinetic resolution of ketoprofen ethyl ester, because of the poor solubility and the high viscosity of 2-ethyl ester, and hence the various surfactants were also tested as additives to enhance the final yield and enantioselectivity in the esterase reaction [5]. In this context, an acetone treatment method was also attempted to improve the enantioselectivity [8]. Therefore, there is an increasing demand for new esterases with high enantioselectivity, activity and stability under suitable conditions for practical use.

We here screened and isolated a *Pseudomon*as strain S34 producing (*S*)-stereospecific esterase with high selectivity to (*S*)-ketoprofen ethyl ester. Cell growth and enzyme production were also investigated under various conditions, and the resulting whole cells and partially purified enzymes were characterized for the development of enzymatic process. Because the isolated enzyme could efficiently produce (*S*)-ketoprofen from the corresponding racemic ethyl ester and the enzyme showed a high conversion yield and enantioselectivity, we anticipate the enzyme to be useful as a biocatalyst for the enzymatic resolution of (*R*, *S*)-ketoprofen.

2. Experimental

2.1. Chemicals

Fast Blue RR, (*R*, *S*)-ketoprofen, α-naphthyl acetate, tributyrin, Triton X-100 and ethoxyethanol were purchased from Sigma. Agarose was from Promega. Acrylamide stock (30%) and protein assay solutions were purchased from Bio-Rad. (*R*, *S*)-Ketoprofen ethyl ester was prepared by a general method for esterification, based on a reported result with slight modification [8]. All other chemicals and solvents were of analytical grade.

2.2. Screening and identification of esterase-producing microorganisms

Soils samples from various ecological niches, such as compost, forest and sewage sludge, were collected and suspended in a buffer (0.85% saline solution) or an enriched medium (tributyrin, 10 g/l; yeast extract, 5 g/l; peptone, 3 g/l). After 1 h incubation, the supernatant was diluted and spread on an agar plate of enriched medium containing 2% agar. Colonies that expressed a lipase or an esterase hydrolyzed tributyrin, thus developing a clear zone around the colony [9]. Colonies showing an activity were picked up and re-spread on a fresh plate. The isolated single colony was inoculated into a 50 ml of the enriched medium containing 25 mM ketoprofen ethyl ester. After 2–3 days cultivation, the culture broths were analyzed by HPLC (Waters) using a chiral column (Chirex Phase 3005, Phenomenex). The strains having a preference for (*S*)-enantiomer with high conversion yield were finally screened and stored at −20 °C for further analyses.

The isolated strain was identified according to the general procedures of Bergey's manual and confirmed by systematic approach of Korea Collection for Type Cultures (KCTC).

2.3. Selection and optimization of production medium

To select an appropriate culture medium, the isolated strain was cultivated at 30 °C in a 500 ml of

baffled flasks containing 100 ml of various culture media supplemented with 25 mM (*R*, *S*)-ketoprofen ethyl ester. Inoculations were prepared by pre-culture of a well-isolated colony in the enriched medium for 24 h. During the cultivations, an aliquot of culture broth was sampled and analyzed for cell growth, enantioselectivity and conversion yields. Carbon and nitrogen sources were also optimized in terms of enzyme activity and conversion yields, based on the identical conditions described above.

2.4. Fractionation of crude extract

Wild-type enzyme was partially purified to analyze the properties of enzyme and small-scale conversion. All fractionation procedures were conducted at 4 °C of cold room. *Pseudomonas* cells from a 2 l culture broth were harvested by centrifugation. The cells were resuspended in a 20 mM of Tris–HCl buffer (pH 8.0) containing protease inhibitors cocktail solution (Sigma), and disrupted by using a sonicator (Cole-Parmer instrument Co.). Cell debris was removed by centrifugation at $14\,500 \times g$ for 30 min, and supernatant was treated with streptomycin sulfate (2%). After centrifugation, the solution was fractionated by adding solid ammonium sulfate, resulting that the enzyme was co-precipitated at 50% saturation. The resulting precipitate was dissolved in a minimal amount of dilution buffer and then dialyzed. The enzyme solutions were loaded on to a column of Resource Q (6 ml) equilibrated with 20 mM Tris–HCl buffer (pH 8.0), in an FPCL system (Pharmacia). The column was washed with 10 volumes of the same buffer and eluted with a linear gradient of 0–0.5 M NaCl. The active fractions were pooled and concentrated using a centricon 10 (Amicon), and then stored for further analyses and conversion experiments.

2.5. Enzyme assay

The reaction mixtures (2 ml) containing 50 mM Tris–HCl, 1% Triton X-100, 5 mM (*R*, *S*)-ketoprofen ethyl ester, and intact cells (1 mg) or partially purified enzymes (30 μg) were incubated for 2 h at 30 °C. The reaction was stopped by heating the solution at 100 °C for 10 min or addition of four volume of absolute ethanol, and resulting precipitate was removed by centrifugation. One unit of esterase activity was defined as the amount of enzyme producing 1 μmol of (*S*)-ketoprofen from corresponding ethyl ester per minute under the specified conditions.

2.6. Characterization of partially purified enzyme

To determine the thermal stability, the enzyme was incubated for 2 h at various temperatures, ranging from 30 to 60 °C, in 0.1 M glycine–NaOH buffer (pH 9.5), and residual activity was measured under standard assay conditions. The pH stability was also investigated by determining the residual activity after the enzyme solution was incubated at 30 °C for 2 h using following buffers. The buffers used were citrate (pH 5–6), phosphate (pH 6–7.5), Tris–HCl (pH 7.5–9), glycine–NaOH (pH 9–10).

To investigate the metal dependency of the esterase, the enzyme was treated with 5 mM EDTA followed by dialysis. The metal ions (1–10 mM) tested in this work added to the EDTA-treated enzyme, and the resulting activity was determined under standard assay conditions. The effect of surfactants and solvents on the enzyme activity was also determined at various concentrations of additives under standard assay conditions.

2.7. Native gel electrophoresis and activity staining

For the native gel electrophoresis, samples were diluted with 0.2 volumes of a native sample buffer containing 50% glycerol. Electrophoresis was performed in a Mighty Small II kit (Hoefer Scientific Co.), according to the procedure of Davis [10]. After gel electrophoresis, the separating gel was soaked into a 100 ml of 50 mM Tris–HCl buffer containing 4.6 mg of α-naphthyl acetate dissolved in a 0.5 ml of ethoxyethatnol. The protein band having the esterase activity was developed by addition of 5 ml of Fast Blue RR (2 mg/ml).

2.8. Small-scale conversion and analyses

The conversion experiments were performed in a total volume of 10 ml containing 25 mM (*R*, *S*)-ketoprofen ethyl ester, 50 mM Tris–HCl (pH 8.0), and 1% Triton X-100 under the standard assay conditions. The concentrations of (*R*, *S*)-ketoprofen ethyl ester, (*S*)- and (*R*)-ketoprofen were determined using high

performance liquid chromatography (Waters). The column and mobile phase used were Chirex Phase 3005 (Phenomenex), methanol containing 30 mM ammonium acetate, respectively. The flow rate was 0.8 ml/min, and the eluent was monitored at 254 nm. Protein concentration was measured by using a protein assay solution (Bio-Rad).

3. Results and discussion

3.1. Isolation of a novel strain with high activity to ketoprofen ethyl ester

Screening of the esterase-producing microorganisms from various ecological niches was carried out by using a selective agar plate. The selective plate for the detection of the enzyme activity contained tributyrin that esterified glycerol by triglyceride. The esterase and/or lipase forming microorganisms convert the water-insoluble tributyrin to soluble glycerol and triglyceride, developing a clear zone around the colonies [9]. The selective plates were incubated at two independent temperatures, 30 and 50 °C, to isolate mesophilc and thermophilic strains, respectively. As the detection method was based on the development of clear zone in the solid plate, the positives (40 strains) were readily detected at moderate incubation temperature (30 °C). The selection in a high incubation temperature was also successful and resulted in some positives strains [11]. The selected strains from high incubation temperature, however, displayed a narrow range of species and a chiral selectivity (*R*-stereospecific), although the low stringent conditions (complex medium, diluted soils, neutral pH, prolonged incubation time) were used. Therefore, we conducted further experiments with isolated strains from moderate incubation temperatures.

In order to screen a potential esterase with high selectivity to ketoprofen ethyl ester, the primarily selected strains were subjected to two separate selections. For a clear comparison, 40 strains, showing a high growth rate and larger clear zone, were compared repeatedly their potential in a plate, and then further screened the strains that had a strict enantioselectivity to (*S*)-ketoprofen ethyl ester. To do so, the screened strains were grown in a test tube containing the enriched medium with 25 mM ketoprofen ethyl ester, and the culture broths were subjected to determine the enantioselectivity. As a result, we chose 28 strains from the primarily screened pool for further analyses (Table 1). Prior to other factors examined, the potential strains showing the high enantioselectivity and conversion yield were considered, which allowed four strains (S13, S14, S21 and S34) as possible candidates for practical use (Table 1). Further comparisons were investigated in the respect to the final conversion yields and enantiomeric excess (ee_p) during the

Table 1
Ketoprofen ethyl ester hydrolysing properties of various microorganisms screened from natural sources

Strain no.	Conversion (%)	$(ee_p)^a$ (%)	Strain no.	Conversion (%)	ee_p (%)
S1	0.3	−100	S14	4.7	53
S2	0.9	100	S16	3.4	0.1
S3	19	−52	S17	41	−44
S4-1	8.4	−52	S18	0.6	100
S4-2	24	−38	S21	5.5	55
S5	21	−26	S23	2.4	3.0
S6-1	42	−52	S24	3.3	−3.2
S6-2	48	−43	S25	21	−68
S7	42	−26	S26	1.3	−100
S9	5.2	7.5	S27	7.9	−52
S10	9.2	−92	S28	1.7	−2.7
S11	6.8	−8.5	S30	41	−42
S12	1.3	0.1	S34	2.6	93
S13	1.3	100	S35	2.3	−27

[a] Enantiomeric excess (ee_p) was calculated based on the following equation: $ee_p = ([(S)\text{-ketoprofen}]-[(R)\text{-ketoprofen}]/[(S)\text{-ketoprofen}] + [(R)\text{-ketoprofen}]) \times 100$.

experiments of small-scale conversions using the whole cell enzymes obtained from a solution culture (200 ml) of four stains. Finally, we isolated a strain S34 as a best candidate for practical application, because the strains exhibited the highest enantiomeric excess (>93% ee_p) even in the various reaction conditions.

3.2. Identification of an isolated strain

By considering the high enantioselectivity and conversion yield, an esterase-producing microorganism was isolated, and further experiments were conducted to identify the strain. The isolated strain was a gram-negative, motile, rod shaped, and showed both catalase and oxidase-positive reactions and no fluorescence. Additionally, the organism utilized glucose, fructose, galactose, sucrose and citrate as carbon sources. Phenotypic analysis and morphological characteristics were strongly suggested the strain as a *Pseudomonas* sp. With these results, the isolated strain was taxonomically identified as a *Pseudomonas* sp. S34 according to Bergey's Manual of Systematic Bacteriology. We also confirmed the results by systematic approaches of Korea Collection for Type Cultures (KCTC), including 16 S rRNA analysis.

3.3. Production medium and culture conditions

After the strain identification, we formulated the production medium for the high level production of the enzyme. Using the isolated strain, we first examined the characteristics of cell growth and esterase production by batch culture with various medium, including *Pseudomonas* minimal and complex medium [12] in which various inducers such as tributyrin, olive oil and (*R*, *S*)-ketoprofen ethyl ester, also included. Among the tested mediums, complex mediums were revealed a more growth and activity than those of defined mediums. The favorable growth was found in a TS media composed of 17 g/l tryptone, 3 g/l soytone, 5 g/l NaCl, 2.5 g/l glucose, 2.5 g/l K_2HPO_4, and the resulting activity was determined to be about 5–7 U/mg protein when the cells were grown in the medium at 30 °C for 2 days.

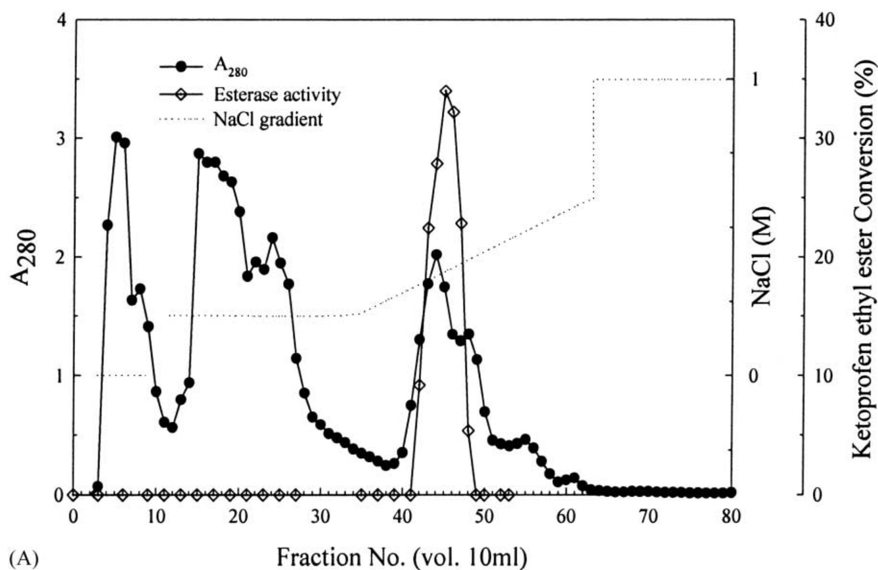
The other factors, such as temperature, inducers, carbon and nitrogen sources, probably affecting the cell growth and enzyme production were further examined [13,14]. First, the effect of various carbon sources, such as glucose, galactose, fructose, lactose, maltose and sucrose, on the production of esterase was investigated and found that the esterase activity was highest in the culture supplemented with glucose as a carbon source. As an alternative carbon source, lactose showed a comparable result to that of glucose. Various nitrogen sources, including yeast extract, peptone, tryptone, soytone, cottonseed flour, soybean flour and casein hydrolysate were also investigated. When the results were compared to that of the control grown in TS medium containing 17 g/l tryptone and 3 g/l soytone as complex nitrogen sources, the comparable results from the tested nitrogen sources at various concentrations were not found.

Some previous results reported that microbial esterases are inducible [15,16], and various potential inducers were also tested with respect to the enzyme activity. Among them tested, no significant inductions were observed with various inducers including lipid and ester compounds [17]. In the case of (*R*, *S*)-ketoprofen ethyl ester, gradual decrease of initial growth and activity were found when the chemical was added into a culture at seeded. The cultivation temperature ranging from 25 to 35 °C revealed a negligible effect on the enzyme production, and therefore we set the cultivation temperature at 30 °C due to its favorable cell growth.

3.4. Fractionation of (S)-ketoprofen ethyl ester-hydrolyzing enzyme

There was a general trends that in a single microbial source various esterases and/or lipases were detected and frequently revealed an activity on the identical substrate, because that a lot of enzymes partly shared a substrate spectrum [18]. Therefore, more than an enzyme could be acted on the (*R*, *S*)-ketoprofen ethyl ester. To exclude the possibility and investigate the functional properties of the enzyme, we intentionally fractionated the enzyme to prepare the faction with an activity to (*R*, *S*)-ketoprofen ethyl ester. For the rapid and accurate fractionation, a preliminary experiment was conducted to choose a simple substrate that readily detected in solid or solution state by activity staining, because that the release of (*S*)-ketoprofen could be monitored tediously by HPLC. When the selected pools (28 strains) by ketoprofen ethyl ester-hydrolyzing activity were compared with primarily screened

40 strains, we found that an ester-hydrolyzing activity was well correlated between (R,S)-ketoprofen ethyl ester and α-naphthyl acetate used as substrates (data not shown). Therefore, we employed alternatively α-naphthyl acetate as a substrate for the detection of enzyme activity during the fractionation procedures. To confirm the hydrolysis of α-naphthyl acetate by the isolated strain exhibiting the activity on the (R,S)-ketoprofen ethyl ester, the activity was detected on the activity staining plate overlaid by a soft agar (0.5%) supplemented with α-naphthyl acetate and Fast Blue RR [19]. As expected, *Pseudomonas* cells



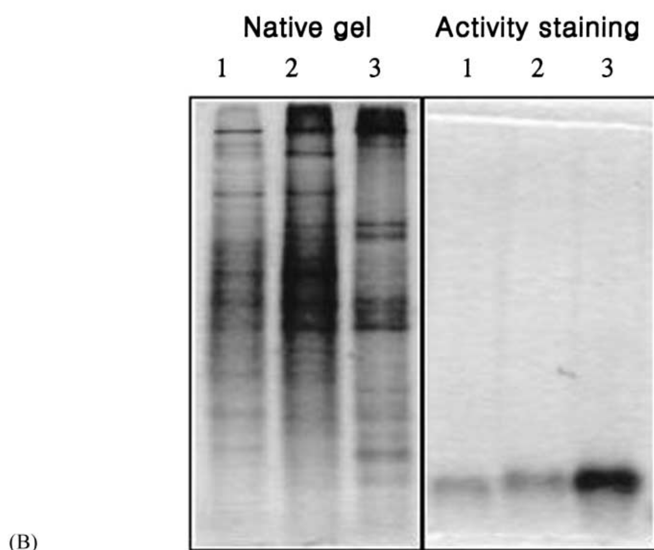


Fig. 1. Fractionation of crude enzyme solutions by ion exchange column chromatography, Resource Q (6 ml). (A) Chromatogram; (B) activity staining of eluted fraction. Each lane contained 30 μg of proteins. Lane 1: crude extract; lane 2: ammonium sulfate fractionation (50%); lane 3: active fractions eluted from Resource Q column chromatography.

exhibited a strong activity on the plate and developed a brown color around colony. Based on these observation, we strongly suggested that the method would be useful for the screening of the enzyme with activity to (R, S)-ketoprofen ethyl ester, even for various purposes, such as strain selection, protein identification and gene cloning.

To fractionate and concentrate the enzyme solution with an activity to (R, S)-ketoprofen ethyl ester, enzyme fractionation was carried out with a 2 l culture broth. There was negligible activity (<3%) in the culture supernatant and thus cell pellets were used for further fractionation. After clarification of the crude lysate by centrifugation, esterase activity was detected mainly in the supernatant fraction, with minor activity (<5%) found in the cell pellet. Crude extracts were further fractionated by co-precipitation with 50% solid ammonium sulfate, and the precipitates was dissolved and dialyzed. The enzyme solution was also fractionated by ultra-filtration using a membrane (cut-off size of 50,000), remaining the most activity in the retentate. The concentrated solution was applied onto a column, Resource Q, and eluted with NaCl gradient ranging from 0 to 0.5 M (Fig. 1). The active fractions, eluted with 0.2–0.25 M NaCl, were pooled and subjected to native gel electrophoresis for activity staining. After the electrophoresis, activity staining was performed by a procedure of Materials and Methods. As shown in Fig. 1B, a distinct band corresponding to an esterase, which was faintly detected in the crude extract, appeared and was not detected in other fractions. About eight-fold of purification was achieved through three successive steps and the resulting activity was determined to be about 48–52 U/mg protein. The resulting enzymes were used for further characterization, without the need for further purification, because we could not detect other activity to (R, S)-ketoprofen ethyl ester in fractionated fractions and also considered the enzyme process with whole cells or crude extracts.

3.5. Enzyme properties

3.5.1. Effects of surfactants and solvents on the enzyme activity

It was also well known that a diverse sets of surfactants, and also solvents, affected the enzyme activities of esterases from various sources, although the extent was quietly different [5,20–22]. The enzyme solutions were pre-incubated (about 10 min) with various water-miscible solvents and surfactants for pre-equilibration. Then, the activity was determined with 25 mM (R, S)-ketoprofen ethyl ester at a concentration of solvents ranging from 5 to 15%, or 1% surfactants. As shown in Table 2, the activity was partly inactivated in the presence of DMF, cyclohexane, DMSO and THF. In these situations, approximately 20–55% of activities were decreased. As a more drastic effect, the serious inhibitions were observed in the presence of hexane and chloroform, although the enantioselectivity was partly increased. Various water-miscible solvents, such as ethanol, butanol, methanol, isopropanol, were also revealed a slightly inhibitory effect on the enzyme activity. In contrast with those, the additions of various surfactants resulted a drastic increase in the conversion yields, and also in the enatntioselectivity, as shown in Table 2. The most promising result, about four-fold of increase, was observed when the Triton X-100 and 165 were supplemented at a concentration of 1%. We further attempted to determine the optimal concentration using the Triton X-100, and found that activity was proportionally increased with increasing concentration of the surfactant and saturated even in a concentration of about 0.6% (Fig. 2).

Table 2
Effects of organic solvent and surfactants on the hydrolysis of *rac*-ketoprofen ethyl ester by an esterase from *Pseudomonas* sp. S34

	Relative activity[a] (%)	ee_p (%)
Control	100	82
DMF	56	100
Cyclohexane	79	97
Hexane	2.7	94
Chloroform	8.9	100
DMSO	45	89
Benzene	39	100
THF	59	94
Triton X-15	180	94
Triton X-45	310	94
Triton X-100	410	95
Triton X-165	430	94
Triton X-305	400	93
Tween 20	86	72
Tween 40	130	81
Tween 60	160	84
Tween 80	140	84

[a] Relative activity shown here was determined in the presence of solvent (10%) and surfactant (1%) under standard assay conditions.

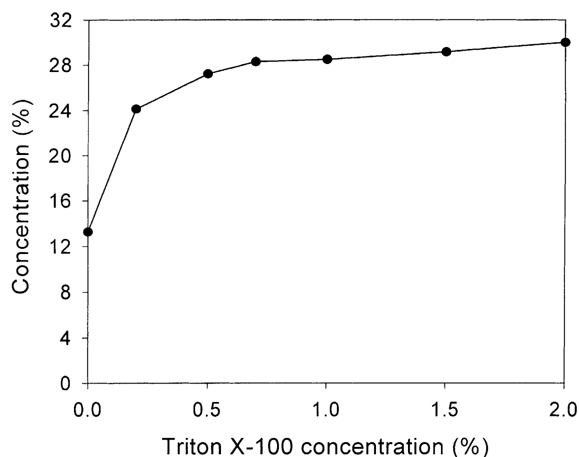


Fig. 2. Effect of Triton X-100 concentration on the conversion rate. The enzyme activity and conversion were determined under standard assay conditions.

3.5.2. Effects of divalent metal ions

It has been generally known that the activity of the ester hydrolase family enzymes is partly or significantly affected by the presence of a cofactor divalent metal ion [23,24]. To determine the specificity with respect to the metal requirement, the enzyme solution (50–75 μg) was first dialyzed with the chelating agent EDTA against metal-free buffer (50 mM Tris, pH 8.0). Enzyme activity was then determined in the presence of different metal ions under standard assay conditions. As a result, a slight decrease (5.5%) in the activity of the EDTA-treated enzyme was observed. The addition of metal ions such as Fe^{2+}, Hg^{2+}, Ag^{2+}, severely inhibited the activity of the enzyme, while a stimulatory effect was not observed in the presence of metal ions or even at a high concentration of metal ions, up to 10 mM. The results strongly suggested that the enzyme activity was not dependent on the metal ions.

3.5.3. Substrate specificity

Substrate specificity was analyzed by the ability to release of *p*-nitrophenyl from various ester compounds [25]. For this purpose, the enzyme fractions were incubated with 5 mM ester derivatives in 3 ml Tris–HCl buffer (50 mM, pH 8.0) at 30 °C for 10 min, and the resulting variance of optical density was monitored spectrophotometrically at 410 nm. As shown in Table 3, the enzyme was found to possess a broad spectrum to ester derivatives available in this study, although no activity for the *p*-nitrophenyl palmitate was observed. Under standard reaction conditions, the esterase showed the highest level of activity toward *p*-nitrophenyl butyrate (72 unit), and a relatively high activity to (R,S)-ketoprofen ethyl ester (52 unit) was also found.

Table 3
Substrate specificity of an ester-hydrolase from *Pseudomonas* sp. S34

Substrate	Specific activity[a] (unit)	Relative activity (%)
p-Nitrophenyl acetate (C2)	59	110
p-Nitrophenyl butyrate (C4)	72	140
p-Nitrophenyl caprylate (C8)	37	71
p-Nitrophenyl palmitate (C16)	ND[b]	
rac-Ketoprofen ethyl ester	52	100

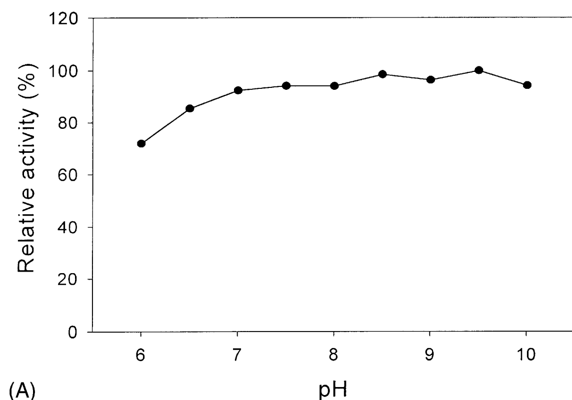
[a] Enzyme activity was determined using the fractionated enzymes in the presence of corresponding substrate (4–5 mM) in a 3 ml of 50 mM Tris–HCl (pH 8.0) buffer. The reaction products were analyzed either by HPLC or spectrophotometer.
[b] ND: not detected.

3.5.4. Effects of pH and temperature

The pH and temperature optimum for the hydrolysis of the ester bond were determined. The temperature dependency of the enzyme showed a highest value at 35 °C, and the pH optimum was revealed at pH 9.5. In order to determine the pH stability, the enzyme solution was incubated for 2 h at different buffers; citrate buffer for pH 5–6, phosphate buffer for pH 6–7.5, Tris–HCl buffer for pH 7.5–9, and glycine–NaOH buffer for pH 9–10, and the remaining activity was determined (Fig. 3A). The activity was stably maintained at pH range from 6.5 to 10, resulting that about 80% of activity was remained in these range. The thermostability was also determined by residual activity analysis after the pre-incubation of enzyme for 2 h at different temperature. The enzyme stable up to 40 °C, and half-life of the enzyme was estimated to be 2 h in 50 °C (Fig. 3B).

3.5.5. Small-scale conversion

To further evaluate the performance of enzyme process, we carried out small-scale conversion with the fractionated enzyme solution, because that the activity of the crude extract and whole cell enzymes was partly fluctuated and showed a minor activity of



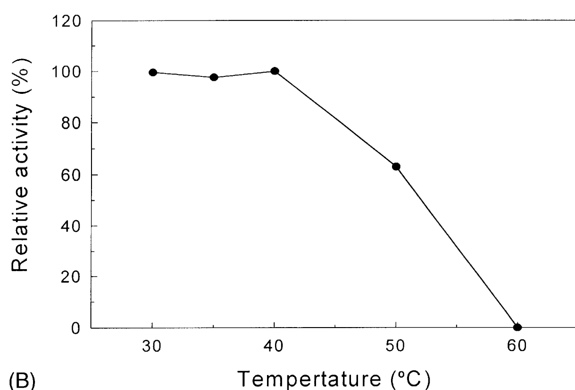


Fig. 3. Effect of pH (A) and temperature (B) on stability of ester-hydrolase from *Pseudomonas* sp. S34. The enzyme solutions were preincubated for appropriated time at the indicated pH and temperature, and the remaining activity was determined under standard assay conditions.

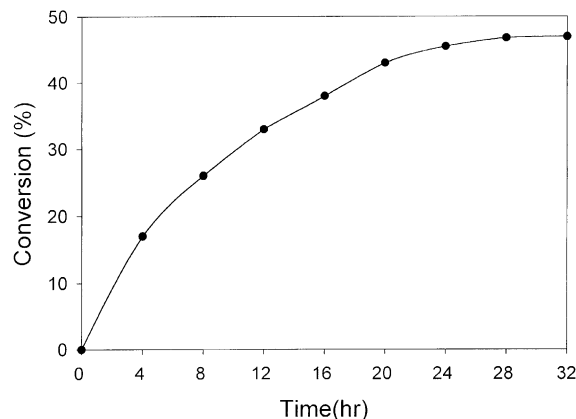


Fig. 4. Small-scale conversion of (*R*, *S*)-ketoprofen ethyl ester by fractionated enzyme fractions. The conversion experiments were conducted according to the conditions described in Section 2.

other esterase. As shown in Fig. 4, when the total activity was fixed at 3500 U, a successful conversion was achieved, about 47% yields with a pure (*S*)-enantiomer (>99%), in a 28 h. As the esterase activity employed in the reaction conditions (25 mM *rac*-ketoprofen ethyl ester, 1% Triton X-100) was increased, the conversion rates were proportionally increased. However, when identical condition was employed in the conversion with crude extracts, a relative lower level of conversion yield (35–38%) and enantimeric excess (86–95%) was revealed, probably due to the contaminated activity of esterase(s) or improper contact between the substrate and the enzyme. Obviously, a conversion by the isolated enzyme was nearly completed and thus suggested that the enzyme may be effectively used as a novel biocatalyst for the synthesis of optically pure (*S*)-ketoprofen.

We report here the screening and partial characterization of an esterase from *Pseudomonas* sp. S34. Recently, an esterase and/or lipase family with high enantioselectivity was identified and found to share quite similar structural and catalytic properties regarding the ester-hydrolyzing properties acting on the similar ester derivatives [11,13,17,19,26,27]. One such member, a lipase OF from *C. rugosa*, is known to act on a ester derivative, (*R*, *S*)-ketoprofen ethyl ester, in the enzymatic resolution of ketoprofen [5,8]. It has been well characterized at the biochemical levels to employ the enzyme for the practical use [5,7,8]. To our knowledge, there was only a report on the other enzyme showed an activity to (*R*, *S*)-ketoprofen ethyl ester, especially in the optically pure production of (*S*)-ketoprofen, however, its biochemical and reaction kinetics were not reported in detail [28]. On the contrast of that from a lipase OF from *C. rugosa* (ee_p 36%), an enzyme exhibited a great value of enantioselectivity (>94%) even with crude extract, thus it provided a promising result that the enzyme was practically applicable for the enzymatic resolution of (*R*, *S*)-ketoprofen ethyl ester. Despite the indisputable observation that it has distinct activity on the (*R*, *S*)-ketoprofen ethyl ester with high enantioselectivity, the physiological function and relationship between the related enzymes are still unknown [19]. Additional physiological functional and biochemical

relationships with other reported esterases, especially with an esterase from *P. fluorescence* [29] and *putida* [30], remained to be elucidated. For this purpose, gene cloning and structural determinations are currently under progress.

4. Conclusions

We describe here the result that an esterase with high selectivity to (*S*)-ketoprofen ethyl ester has been screened and identified. The enzyme hydrolyzed the (*S*)-ketoprofen ethyl ester to (*S*)-ketoprofen in the stereo-specific manner (>99% ee_p), and the enzyme did not require a cofactor, such as metal ions. Additionally, the enzyme was significantly activated in the presence of a surfactant Triton X-100, even at a concentration of 0.6%, without the loss of enantioselectivity. Currently, the practical application of an esterase or a lipase in enzymatic resolution of (R,S)-ketoprofen ethyl ester to (*S*)-ketoprofen is limited by the low enantioselectivity of the enzymes, although the partial success was achieved by addition of an emulsifier. The novel enzyme, with high conversion yield and enantioselectivity, from *Pseudomonas* sp. S34, therefore, was strongly suggested that the enzyme might be effectively used as a novel biocatalyst for the synthesis of optically pure (*S*)-ketoprofen.

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