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A new isolate *Bacillus stearothermophilus* JY144 expressing a novel esterase with high enantioselectivity to (*R*)-ketoprofen ethyl ester: strain selection and gene cloning

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Abstract

In order to obtain novel strains that hydrolyzed the *rac*-ketoprofen ethyl ester to an enantiomer of ketoprofen, we enriched strains from broad ecological niches in which the thermostable enzymes were ubiquitously found. The isolated strains were first analyzed to detect the ester-hydrolyzing activity by using a selective plate, and then further screened by activity on the (*R*,*S*)-ketoprofen ethyl ester. Twenty-six strains were screened primarily and further compared for the ethyl ester-hydrolyzing activity in terms of conversion yield and enantioselectivity. Consequently, a strain JY144 was isolated as a novel strain that produced a (*R*)-stereospecific esterase with a high stability and systematically identified as a *Bacillus stearothermophilus* JY144. The enzyme was indeed stable at a broad range of pH (6.0–9.5) and temperature, up to 65 °C. The optimal temperature and pH for enzymatic conversion were 50 °C and 9.0, respectively. Since the wild-type strain showed a poor cell growth and enzyme activity, we further attempted the cloning of esterase gene into *Escherichia coli*, with a simple and rapid strategy for selecting the positive clones, and determined its primary structure. The cloned gene had an open reading frame (250 amino acids) with a calculated molecular mass of 27.4 kDa. The primary structure showed a relatively high homology (38–52%) to probable esterases unidentified to date, and thus presumed as a novel family enzyme. In whole cell conversions, the recombinant enzyme had a superior potential than the parent strain, and it could completely convert the *rac*-ketoprofen ethyl ester to (*R*)-ketoprofen, with an optical purity of 99%.
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1. Introduction

Chiral resolution of racemic chemicals composed of alternative enantiomers by using a stereospecific enzyme is gaining a lot of attention and thus steadily increasing in the fine chemistry and pharmacology for practical use [1,2]. The stereospecific production of an enantiomer was demanded critically because its pharmacological activity was linked with an enantiomer, but other counterpart is biologically inactive or had a negative effect [3]. Given chemicals to be resolved, various enzymes with high stereoselectivity to an enantiomer, including hydrolases, dehydrogenases and oxido-reductases, are intensively studied in a variety of reactions [4–7]. Among them, esterases are also well documented on the bio- and stereochemical

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aspects by many researchers who mainly focused on the practical applications of wild-type or engineered enzymes with desired properties [8].

Esterases, also including lipases, catalyze diverse sets of ester compounds to their hydrolytic product on a large number of natural substrates or unnatural derivatives [5,8,9]. Industrially attracting enzymes often exhibit a high enantio- and regioselectivity, as well as broad substrate specificity [8,10–12]. Therefore, a wide range of (carboxylic) ester compounds are currently subjected to the chiral resolution using the specialty enzymes with high selectivity and hence, applications of these enzymes are widely found in the food, detergent, pharmaceutical and chemical industries. In contrast to the fact that various lipases are commercially available in both natural and recombinant enzymes, a few of esterases with desired properties are cloned and available in resulting recombinant forms [13–15]. Especially, there was no report on the cloning of the enzyme with high stereoselectivity to ketoprofen ethyl ester as a target substrate [11].

The chiral specific production of optically pure ketoprofen had been accomplished by resolution of corresponding racemic mixtures [16,17]. Among the reported enzymes, *Candida rugosa* lipase and *Trichosporon* esterase have been known to have a relatively high activity to the (*S*)-enantiomer and therefore currently investigated for the stereospecific resolution of (*R*,*S*)-ketoprofen ethyl ester [18,19]. We have also attempted to screen the stereospecific esterase with high activity and selectivity to ketoprofen ethyl ester, and recently reported a strictly (*S*)-stereospecific enzyme from *Pseudomonas* sp. S34 [11]. The other enantiomer (*R*)-ketoprofen, used for preventing periodontal disease as a toothpaste supplement [20], was also converted stereospecifically from their corresponding esters or amide of ketoprofen [21,22], and from the ethyl ester form of ketoprofen [23].

We here further screened an enzyme with high stability, and also with high selectivity to an enantiomer of ketoprofen, from a pool of thermophlic strains, by considering the typical problems of poor solubility and high viscosity of 2-ethyl ester. We consequently isolated a *Bacillus* strain JY144 producing a thermostable esterase with high selectivity to (*R*)-ketoprofen ethyl ester. Cell growth and enzyme production were also investigated under various conditions and characterized for the development of enzymatic process. Since the enzyme could not be efficiently produced from the isolated strain, we cloned and overexpressed the gene encoding an esterase from *Bacillus stearothermophilus* JY144 in *Escherichia coli*. The resulting esterase gene was analyzed and compared with those of related enzymes. We also here established a simple and rapid screening procedure for the isolation of positive clones that expressed the ketoprofen ethyl ester-hydrolyzing enzymes.

2. Experimental

2.1. Chemical and enzymes

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 BioRad. Restriction enzymes, Klenow fragment and T4 DNA ligase were purchased from KOSCO (Korea). A thermophilc DNA polymerase for PCR was from New England Biolabs. (*R*,*S*)-Ketoprofen ethyl ester was prepared by a general procedure for esterification, based on a reported procedure with slight modifications [11]. All other chemicals and solvents used were of analytical grade.

2.2. Screening and identification of ketoprofen ethyl ester-hydrolyzing microorganisms

Hundreds of samples were collected from various ecological niches, such as compost, forest, hot spring and sewage sludge, and suspended in a 50 mM phosphate buffer (0.85% saline solution) or an enriched TS medium formulated previously [11]. After 5–7 h incubation at 50 °C, the debris and organic precipitates were removed by a brief centrifugation, and the supernatant was diluted and spread on an agar plate of enriched medium containing 1% tributyrin and 2% agar. With a clear zone around the colony, strains showing an activity were primarily selected and re-spread on a fresh plate. The isolated single colony was inoculated into a 50 ml of the TS medium with or without 10 mM ketoprofen ethyl ester. After 24 h cultivation at 50 °C and 180 rpm, the culture broth was centrifuged. Both cell pellet and supernatant were separately harvested and then analyzed

the hydrolytic activity to *rac*-ketoprofen ethyl ester. The strains having a preference for an enantiomer with high conversion yield were finally screened and stored at −20 °C for further analyses.

The isolated strain was systematically identified by general procedures of Bergey's manual and confirmed by 16 S rRNA sequencing, and deposited in the Korea Collection for Type Culture (KCTC) and designated as *B. stearothermophilus* KCTC 3775.

2.3. Cell growth and enzyme production of Bacillus stearothermophilus JY144

To select a culture medium favorable for cell growth and enzyme activity, the isolated strain was cultivated at 50 °C in baffled flasks (500 ml) containing 100 ml of various culture media supplemented with or without 10 mM (*R*,*S*)-ketoprofen ethyl ester. Inoculating cells were prepared by pre-culture of a well-isolated colony in the enriched medium (10 ml) for 24 h. During the cultivation, an aliquot of culture broth was withdrawn and analyzed for cell growth, enzyme activity and enantioselectivity. Carbon and nitrogen sources were also analyzed for their influences on the enzyme activity and conversion yield, based on above conditions.

2.4. Temperature and pH dependency of the enzyme

To determine the thermostability, the protein solution of crude extracts was incubated for 2 h at different range of temperature (30–60 °C) in 0.1 M Tris–HCl buffer (pH 8.5), and residual activity was determined under standard assay conditions. The pH stability was also determined by measuring the remained activity after preincubation at 30 °C for 2 h using following buffers: citrate (pH 5–6), phosphate (pH 6–7.5), Tris–HCl (pH 7.5–9), glycine–NaOH (pH 9–11). The temperature and pH optima were also determined under identical conditions.

2.5. Strain and plasmid for esterase gene cloning

E. coli XL1-Blue, a general host for the cloning and propagation of a recombinant gene, was used for the library construction and expression of the cloned enzyme. Plasmid pBluescript II SK, used for library construction, was obtained from Stratagene. *E. coli* cells were grown at 37 °C in Luria–Bertani (LB) broth supplemented with ampicillin (50 μg/ml) when needed.

2.6. DNA library construction and primary screening

Total chromosomal DNA was isolated from *B. stearothermophilus* JY144 by using a genomic DNA purification kit (Promega) and manipulated by using standard recombinant DNA techniques [24]. The purified genomic DNA was partially digested with restriction enzymes *Sau*3AI or *Mbo*I, and the resulting DNA fragments were fractionated on agarose gel and purified by using a DNA clean up system (Promega). For the library construction, the eluted fragments were cloned into a *Bam*HI site of pBluescript II SK.

E. coli transformants were grown at 37 °C for 24–30 h in a typical LB medium supplemented with ampicillin (50 μg/ml). After replica plating, both the original and new plates were further incubated overnight at 37 °C, and one plate was used for primary screening. The positive clones were screened by activity staining of cells grown in LB plate with an overlaid soft agar (0.6%) containing Fast Blue RR (15 μg/ml) and α-naphthyl acetate (45 μg/ml). The α-naphthyl acetate (4.5 mg) was first dissolved in ethoxyethanol (0.5 ml) and then added into soft agar solution. The positive clones rapidly (<5 min) developed a deep brown color around colony [11,13].

2.7. DNA sequencing and computer analyses

Nested deletion sets of the cloned DNA were constructed from both ends by digestion with restriction enzymes, and nucleotide sequences of both strands were determined using double-stranded DNA as a template with the universal primers T3 and T7.

The amino acid sequence deduced from the esterase gene of *B. stearothermophilus* JY144 was aligned by hierarchical clustering of the individual sequences based on the pair-wise similarity scores. The related enzymes and their sequences were searched and compared with various enzymes from other sources using the BLAST network service at the National Center for Biotechnological Information (NCBI). The identity and homology of amino acid sequences shared in related enzymes were analyzed by Clustal W program [25]. Further analyses were performed by visual

inspection or an ORF finder (NCBI) for proofing the sequences.

2.8. Native gel electrophoresis and activity staining

For the activity staining on the native PAGE, protein samples were mixed with 0.2 volumes of a native sample buffer containing 50% glycerol and revolved on a 12% acryl amide gel under non-reducing conditions, according to the procedure of a previous report [11]. After gel electrophoresis, the separating gel was washed twice with a 20 ml of 50 mM Tris–HCl buffer and then soaked into the same buffer (100 mM) containing 4 mg of α-naphthyl acetate dissolved in a 0.5 ml of ethoxyethatnol. The protein band of active enzyme was developed by addition of 2–3 ml of Fast Blue RR (2 mg/ml), and then sliced. The activity was further confirmed by using the sliced gel and ketoprofen ethyl ester as a substrate.

2.9. Small-scale conversion

The conversion experiments in small scale were performed in a total volume of 10 ml containing 10 mM (*R*,*S*)-ketoprofen ethyl ester, 50 mM Tris–HCl (pH 8.0), and 1% Triton X-100 under the standard assay conditions. For a clear comparison, the wild-type and recombinant cells were prepared separately and compared the conversion profiles in terms of reaction rate, conversion yield and enantioselectivity.

2.10. Enzyme assay and analyses

The reaction mixtures (2 ml) containing 50 mM Tris–HCl, 0.5% Triton X-100, 5 mM (*R*,*S*)-ketoprofen ethyl ester, and intact cells (3–5 mg) or crude extracts of the enzymes (0.2–0.5 mg) were incubated for 2–5 h at 50 °C. The reaction was stopped by heating the solution at 100 °C for 15 min or addition of 4 volumes 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 ketoprofen from corresponding ethyl ester per minute under the specified conditions.

The concentrations of (*R*,*S*)-ketoprofen ethyl ester, (*R*)- and (*S*)-ketoprofen were determined by peak areas of retention time at 4.8, 14.3 and 15.7 min, respectively, using high performance liquid chromatography (Waters). The column and mobile phase used were Chirex Phase 3005 (Phenomenex), methanol containing 30 mM ammonium acetate, respectively. At a constant flow (0.8 ml/min), the eluent was monitored at 254 nm. Protein concentrations were measured by using a protein assay solution (BioRad).

3. Results and discussion

3.1. Strain selection by a stereospecific hydrolyzing activity to ketoprofen ethyl ester

By considering the low solubility and high viscosity of *rac*-ketoprofen ethyl ester, we screened thermostable esterases from various ecological niches such as soils, sewages, contaminated water and composts, since it was frequently found that thermostable enzymes also rather resisted to organic solvents than mesophilic counterparts. Using the well-isolated colony from enriched cells at high incubation temperature, the ester-hydrolyzing activity was first analyzed by using a selective agar plate, according to the previous procedure [11]. The selective plates were incubated at 50 °C, to isolate the strains that expressed the enzyme with enhanced stability. Based on the developed clear zone in solid plates, approximately 300 positive strains were readily detected. The primary selection was also performed in an enriched medium supplemented with (*R*,*S*)-ketoprofen ethyl ester (5–25 mM) as an inducer. However, in that case, more narrow strain pools were obtained than that conducted without an inducer, and it was not much improved even in the low stringent conditions attempted here (complex medium, diluted soils, neutral pH, prolonged incubation time).

In order to screen a potential esterase with high selectivity to ketoprofen ethyl ester, the primarily selected strains were subjected to further selections. For a clear comparison, 300 strains, showing a high growth rate and distinct clear zone, were compared repeatedly for their potential in a plate, and then further screened the strains that had a strict selectivity to an enantiomer. For that purpose, possible strains were grown in an enriched medium (50 ml) with or without 10 mM ketoprofen ethyl ester, and the grown cells were harvested and subjected to the analyses of conversion yield and enantioselectivity. As a result, we chose 26 strains

Table 1
Ketoprofen ethyl ester-hydrolyzing properties of primarily screened thermophiles

Strain number	Conversion (%)	ee_p (%)
JY84	3.6	−100
JY93	15.4	−100
JY104	17.3	−100
JY1118	7.9	−100
JY128	8.0	−43
JY1212	5.4	−100
JY1213	10.0	−100
JY1215	12.2	−70
JY132	8.0	−100
JY133	6.8	−100
JY136	10.2	−100
JY144	38.7	−100
JY151	5.5	−100
JY152	8.2	−100
JY154	10.0	−100
JY167	8.5	−100
JY184	19.3	−100
JY187	15.3	−100
JY204	13.0	−100
JY205	4.9	−100
JY2111	18.9	−100
JY213	3.3	−100
JY232	23.8	−100
JY244	20.0	−100
JY252	8.3	−100
JY258	11.0	−100

Enatiomericexcess (ee_p) = {[(*S*)-ketoprofen − (*R*)-ketoprofen]/[(*S*)-ketoprofen + (*R*)-ketoprofen]} × 100.

from the primarily screened pool for further analyses (Table 1). Next, the potential strains showing the high enantioselectivity and conversion yield were finally compared, allowing six strains (JY93, 104, 144, 184, 2111 and 232) as possible candidates for practical use. As shown in Table 1, the enantioselectivity was severely biased to a preference for (*R*)-enantiomer. Only two strains JY128 and 1215 showed a detectable activity to (*S*)-enantiomer, but the other (*R*)-form also appeared in whole cell reactions. These results may provide a possibility that the (*S*)-stereospecific enzyme was scarcely distributed and thus could not easily be obtained from a pool of thermophilic strains, because no enzyme activity to (*S*)-enantiomer was found with re-experiment of primary screened pool and also with typical strains from a culture collection.

Further comparisons of six strains were investigated in respect to the cell growth, activity and stability of whole cell enzymes. Finally, we isolated a strain JY144 as a best candidate for practical application, because the strains exhibited the highest enantiomeric excess (>99% ee_p) and conversion yield (∼40%) even in a broad range of reaction temperature (40–60 °C), and also retained a strict enantioselectivity at various conditions.

3.2. Identification and culture conditions of an isolated strain

After the strain selection, further experiments were carried out to identify the strain. The isolated strain was a gram-positive, motile, rod shaped, and showed an endospore as a typical feature in the related strains. Additionally, the organism utilized glucose, xylose, maltose, manitol and mannose as carbon sources, and a detectable growth was founded in a broad range of temperature from 40 to 65 °C. Phenotypic analyses and morphological characteristics strongly suggested the strain as a *B. stearothermophilus*. Based on these results, the isolated strain was taxonomically identified as a *B. stearothermophilus* JY144 according to Bergey's Manual of Systematic Bacteriology, which was further confirmed by analysis of the 16 S rRNA sequence.

By considering the factors and conditions that were used for screening the esterase-producing microorganisms with high selectivity to ketoprofen ethyl ester, we formulated the production medium for high-level production of the enzyme. We first examined the characteristics of cell growth and esterase production by batch cultures with various media, including *Bacillus* minimal and complex medium [26] in which various inducers such as lipids and ester compounds were also included. Among the tested media, the favorable growth was found in a pre-formulated TS media [11] 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 0.07–0.1 U/mg protein when the cells were grown in the medium at 50 °C for 25–30 h. There were no comparable results obtained from tested media, including a well-defined medium for similar strains [27].

The other factors, such as temperature, inducers, carbon and nitrogen sources, probably affecting the cell growth and enzyme production were also examined. First, the effect of various carbon sources, such as

glucose, galactose, fructose, lactose, maltose and sucrose, on the production of esterase was investigated. Next, various nitrogen sources, including yeast extract, meat extract, peptone, tryptone, soytone and casein hydrolysate were also investigated. All the cases tested here revealed no significant improvement in the enzyme activity, although the favorable cell growth was resulted in a medium supplemented with glucose and yeast extract, as carbon and nitrogen sources, respectively.

From the subsequent experiments with probable inducers and metal ions [28,29], we also could not find an effective additive that stimulated the enzyme production and thus resulted in a high activity of the enzyme. In particular, an initial growth and activity were severely decreased when the cells were grown in a culture supplemented with the (*R*,*S*)-ketoprofen ethyl ester. Since some related enzymes can traverse the cell membrane and thus liberated from the cell, each activity was analyzed with culture supernatant and separated whole cell enzyme [27]. The marginal but considerable activity, about 25–30% of the whole cell enzyme, was also revealed in culture supernatant. The cultivation temperature ranging from 40 to 65 °C showed a minor effect (1.2–1.5-fold) on the specific activity of enzyme, and therefore we routinely cultivated the cell at 50 °C for favorable cell growth. Therefore, these results strongly suggested that the high-level expression of the enzyme could not readily be obtained from wild-type strain in normal conditions, although the factors affecting the cell growth and enzyme production still remained for further consideration.

3.3. Temperature and pH dependency of the enzyme

The results that, described above, revealed a low cell mass and enzyme activity led us to clone the gene into a general host *E. coli* in which the high cell mass and enzyme production were easily achieved due to its susceptibility to induction. Prior to the experimental progress, we preliminarily investigated the temperature and pH dependency of the enzyme, which was a principal criterion for strain selection and thus, further experiments. As expected, the esterase was indeed functional at high incubation temperature. The enzyme showed the highest activity at 55 °C, and more than the half of activity (60%) was also retained even after the preincubation of the enzyme at 70 °C for 2 h (Fig. 1A). Additionally, the enzyme was also stable at a broad range of pH from 6.0 to 9.5, but more favorable in acidic pH. The enzyme showed the maximum activity at pH 10 (Fig. 1B).

3.4. Library construction and primary screening

The library construction for the esterase gene cloning was performed by a typical procedure of general protocol [24]. The cells grown in TS medium were harvested in mid-exponential phase and their genomic DNA was separated by using a kit or a reported protocol for the similar strain [30]. *B. stearothermophilus* JY144 chromosomal DNA was partially digested with *Sau*3AI, and the fragments ranging from 1 to 5 kb were recovered from agarose gel. The resulting fragments were then ligated into the *Bam*HI site of pBluescript II SK and transformed into *E. coli* XL1-Blue.

As reported previously, for the rapid and simple detection of the esterase with high selectivity to ketoprofen ethyl ester, an alternative substrate readily detected in solid or solution state by activity staining is highly required, because that the release of (*R*)- and (*S*)-ketoprofen could only be monitored tediously by HPLC [11,22]. In addition, *E. coli* cells showed a confused phenotype, likely as an activity hallow, on the tributyrin plate in a prolonged incubation, often misleading the results unless carefully checked. In this context, we have been suggested α-naphthyl acetate as a possible substrate for the activity indicator at the screening step [11]. To confirm the hydrolysis of α-naphthyl acetate by the (*R*,*S*)-ketoprofen ethyl ester-hydrolyzing enzyme, the crude extracts were loaded and resolved on a native gel, and then activity-stained with α-naphthyl acetate and Fast Blue RR (Fig. 2). As shown in Fig. 2, the protein solution eluted from the activity-stained position exhibited a strong activity on the (*R*)-ketoprofen ethyl ester as expected, and thus we were strongly convinced that the activity staining with α-naphthyl acetate and Fast Blue RR can be useful for the screening of the enzyme with activity to (*R*,*S*)-ketoprofen ethyl ester, especially for the selection of positive clones from library pools.

Since the ester-hydrolyzing activity was well correlated between (*R*,*S*)-ketoprofen ethyl ester and

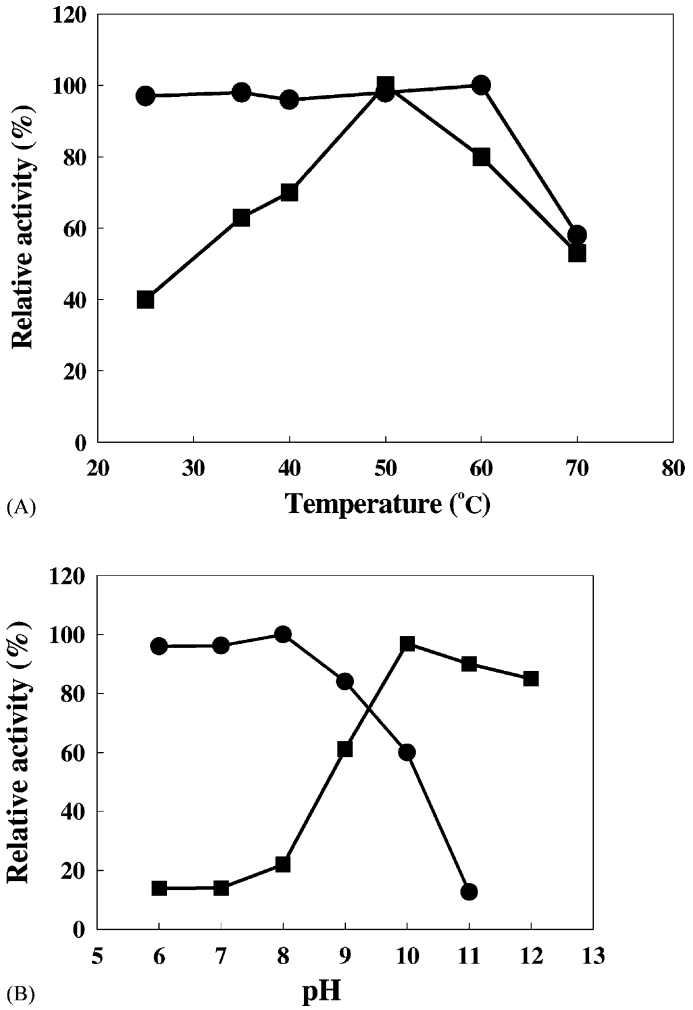


Fig. 1. Effects of temperature (A) and pH (B) on the activity (■) and stability (●) of the esterase from *Bacillus stearothermophilus* JY144. To determine the stability, the enzyme solutions were preincubated for 2 h at the indicated pH and temperature, and the remaining activity was determined under standard assay conditions.

α-naphthyl acetate used as substrates, the recombinant clones carrying an inset were initially screened by activity staining on the typical LB medium. After a prolonged incubation (25–30 h), the plates were overlaid by a soft agar containing α-naphthyl acetate and Fast Blue RR. We screened positive clones based on the developing color, a bright brown, around the colony within 5–10 min. In these situations, the *E. coli* transformants carrying the control plasmid revealed no color change.

3.5. Physical map and DNA sequencing

To obtain appropriate clones for further analyses, the transformants were subjected to two successive screenings. Primarily, seven clones that developed a distinct color were selected from a library pool (12,000 clones), and then further compared the enzyme activity to ketoprofen ethyl ester. These results were confirmed by re-transformation of isolated plasmids into a fresh competent cell. As a result, one

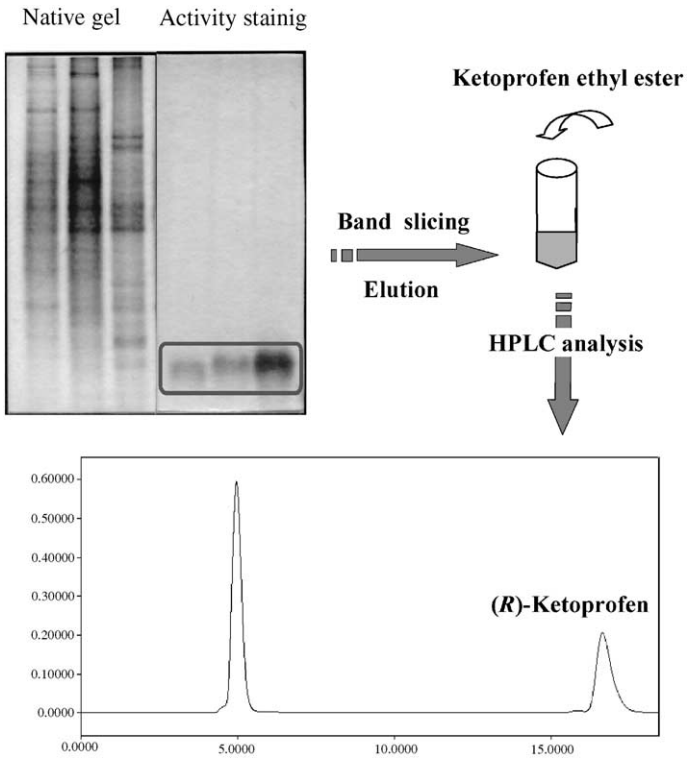


Fig. 2. Enzyme activity correlation between two substrates, α-naphthyl acetate and ketoprofen ethyl ester. After native gel electrophoresis and then activity staining with α-naphthyl acetate, the eluted protein solution from a stained band was assayed using ketoprofen ethyl ester as a substrate.

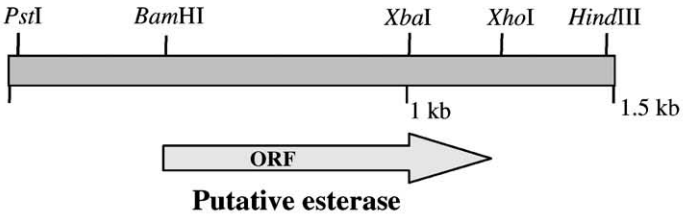
clone showing the highest activity was screened and found that the activity was about 15–20-fold higher than that of the wild-type enzyme, with a strict preference to (*R*)-enantiomer (>99% ee_p) as revealed in the original strain. From the selected clone, the harboring plasmid (pBSEST1) was purified and analyzed with restriction endonucleases. The esterase activity was found to be associated with a 1.5 kb of insert (Fig. 3A). The subclone that contained the insert in the opposite direction to pBSEST1 produced a similar level of enzyme, indicating the gene controlled by its own promoter. The plasmid pBSEST1 was chosen as the sequencing template for further analyses, without the need for further size reduction, due to its proper size for sequencing and sufficient expression.

In order to find a possible ORF encoding an esterase, the nucleotide sequence of the 1.5 kb insert form pBSEST1 was completely determined on both strands and showed the whole size to be 1424 bp. Upon scanning on the GenBank data base, a distinct ORF with deduced amino acid sequence similar to those of a previously characterized or probable esterases was found (Fig. 3A). Consistent with this, further analyses that tried to find an ORF by other software tool, such as ORF finder, yielded an identical result.

3.6. Characterization of the esterase gene from B. stearothermophilus JY144

The predicted ORF consisted of 753 bp, and its deduced amino acid sequences from putative esterase gene revealed an open reading frame of 250 amino acid residues with a calculated molecular mass of 27.4 kDa and an isoelectric point of pH 5.5 (Fig. 3B). As general feature, the translation began with an ATG and ended with a TAA. The calculated molecular mass from the predicted region was in good agreement with that determined for the enzyme by SDS-PAGE (data

(A)



(B)

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                                                                GTGGATCGCTTTTCCATCCTGATTCTTCTTTATTTTCTTC
    ATTTTGAGTTCTACGCCATATCTCGCCATAGACCCTCTTCAACTTTACGTTCATATTCGTTTGTCGGATTATTATAACC
    CGTGGTGCTAGATAAACTCAAACAAGCATAAAAATAGCCCTTGCATGAGGATCCCCTTAAAATGAAAGTGCAACCAACC
    ATTAAAAAGGAGGAAAAAAGAGGTGCGGCGGCGAATATAGTGAGAGGAACGGATTTGACTAGATAAGGGGGAGAGGAAG
  1 ATG AGC GAA CAA TAT CCG GTG CTC TCG GGC GCC GAG CCG TTT TAC GCC GAA AAC GGG CCG
  1 M   S   E   Q   Y   P   V   L   S   G   A   E   P   F   Y   A   E   N   G   P

 61 GTC GGG GTG CTG CTC GTG CAC GGA TTC ACC GGC ACG CCC CAC AGC ATG CGC CCG CTC GCT
 21 V   G   V   L   L   V   H   G   F   T   G   T   P   H   S   M   R   P   L   A

121 GAA GCG TAT GCG AAA GCC GGC TAT ACC GTT TGC CTG CCG CGC TTA AAA GGG CAC GGA ACG
 41 E   A   Y   A   K   A   G   Y   T   V   C   L   P   R   L   K   G   H   G   T

181 CAT TAC GAA GAC ATG GAA CGG ACG ACG TTC CAC GAT TGG GTC GCC TCG GTC GAA GAA GGA
 61 H   Y   E   D   M   E   R   T   T   F   H   D   W   V   A   S   V   E   E   G

241 TAT GGA TGG CTG AAA CAA CGA TGC CAA ACC ATT TTT GTC ACC GGG CTG TCG ATG GGC GGG
 81 Y   G   W   L   K   Q   R   C   Q   T   I   F   V   T   G   L   S   M   G   G

301 ACG CTC ACG CTT TAT TTG GCG GAA CAT CAC CCA GAC ATC TGC GGC ATC GTG CCG ATT AAC
101 T   L   T   L   Y   L   A   E   H   H   P   D   I   C   G   I   V   P   I   N

361 GCC GCT GTC GAC ATC CCG GCC ATC GCC GCC GGG ATG ACG GGC GGG GGC GAG CTG CCG AGG
121 A   A   V   D   I   P   A   I   A   A   G   M   T   G   G   G   E   L   P   R

421 TAT CTG GAT TCG ATC GGT TCG GAC TTG AAA AAT CCG GAT GTG AAA GAG CTG GCA TAC GAG
141 Y   L   D   S   I   G   S   D   L   K   N   P   D   V   K   E   L   A   Y   E

481 AAA ACG CCG ACC GCT TCG CTT CTT CAG CTG GCT AGG CTG ATG GCA CAG ACA AAA GCG AAA
161 K   T   P   T   A   S   L   L   Q   L   A   R   L   M   A   Q   T   K   A   K

541 CTC GAT CGC ATC GTC TGT CCG GCG TTG ATT TTT GTC TCC GAC GAA GAT CAC GTC GTG CCG
181 L   D   R   I   V   C   P   A   L   I   F   V   S   D   E   D   H   V   V   P

601 CCG GGA AAC GCC GAC ATC ATC TTT CAA GGC ATT TCA TCG ACG GAG AAA GAG ATC GTC CGC
201 P   G   N   A   D   I   I   F   Q   G   I   S   S   T   E   K   E   I   V   R

661 CTC CGA AAC AGC TAC CAT GTG GCG ACG CTC GAT TAC GAC CAA CCG ATG ATT ATT GAA CGG
221 L   R   N   S   Y   H   V   A   T   L   D   Y   D   Q   P   M   I   I   E   R

721 TCT CTC GAA TTT TTC GCC AAG CAC GCC GGA TAA 753
241 S   L   E   F   F   A   K   H   A   G   *   250
    AACGAATCGGTATATGGCTGATCCTGTTCAAAGGAGGCCGAAAAGCCTTGATAGATCAACATTTCTAGAGGGAGGATGG
    CTATGGGGAACATTCAACCAAGGCGGTTGATGACGGCAAGCGGCCGGCCGATTGTCGTACGGACGGCATCACCTGAGGA
    TGCCGAGCGTGTCGTTGCGTTTGTGAAGGCGGTGGCGGCGGAAGCGCCGTATTTGCTGACGACGGAAGCGGAAGTGGTG
    ACGACAAGCGAGCGGCAAAGGCAATGGCAGCTTATCGATACCGTCGAcCTCGAGGGGGGGCCCGGTACCCAAT
```

Fig. 3. Physical map and nucleotide sequence of the cloned DNA fragment from *Bacillus stearothermophilus* JY144. In the physical map (A), open arrow indicates the proposed direction and extension of the putative ORF. The amino acid sequence was deduced from the putative esterase gene (B), and the consensus sequences conserved in the related family of enzymes are marked boldly. The putative ribosome binding site is shown in gray box.

not shown). Additionally, the ATG codon is in a favorable context for translation initiation because of its location near the downstream region of putative ribosomal binding site (GGAGAG). At the upstream region of the ORF shown in Fig. 3B, possible sequences corresponding to the typical elements of *Bacillus* promoters were also found. The sequence and organization of these elements were quite similar to those of *E. coli*, and therefore this is the reason that the enzyme was expressed moderately by its innate promoter in *E. coli*. The result also provided a possibility of a constitutive expression without promoter change [30]. For the catalytic function, the whole ORF was entirely required for functional expression of the enzyme with intrinsic properties, because the nested deletion sets from both ends, spanning the predicted esterase gene, apparently resulted the inactive enzyme.

The homologous enzymes and/or proteins to the esterase from *B. stearothermophilus* JY144 were searched on and selected from the protein data bank by a basic local alignment search tool (BLAST), in order to obtain the insight into the structure and function relationships among the related enzymes. BLAST search showed a close relationship between JY144 esterase and putative enzymes from various sources. The best scores (38–52%) were observed with those of esterases, an identified carboxyl esterase (Q06174) from *B. stearothermophilis* [31] and probable esterases or lipases from *Streptomyces coelicolor* (T35504), *B. subtilis* (NP-391242), *Thermotoga maritima* (NP-228828), *B. halodurans* (NP-244421), *Listeria monocytogenes* (NP-465973) and *Staphylococcus aureus* (NP-371303). Most of the enzymes referred here, with an exception [31], still uncharacterized to their function and thus are not reported to date. As shown in Fig. 4, we further aligned the amino acid and nucleotide sequences of these esterases, including JY144 esterase, and found that the open reading frames had a similar secondary structure content and structural organization throughout the entire region, with values close to those of identical or counterpart enzymes. The consensus sequences, H-G and G-X-S-X-G, shared commonly in the majority of esterases and lipases as catalytic motifs were also identified at the identical positions [13–15]. These results provided a possibility that the other esterases may be also able to act on the *rac*-ketoprofen ethyl ester, although the activity has not yet been reported to date. At the molecular level, thus, these enzymes may provide a novel biocatalyst for enzyme-mediated chiral resolution of ethyl ester, and also new insights into functions as novel mechanisms involved in the detoxification of xenobiotics preferentially hydrolyses triglycerides with short chain fatty acids rather than long chain ones [31].

3.7. Small-scale conversion with recombinant enzymes

To evaluate the potential of the recombinant enzyme for practical use in the production of optically pure (*R*)-ketoprofen, we conducted a small-scale conversion. As a preliminary experiment, we first compared the expression level and enzyme activity between the wild-type and recombinant enzymes, each of them was prepared from solution cultures at 50 and 37 °C, respectively, for 22 h without induction. The specific activity of recombinant enzymes was about seven-fold higher than that of the wild-type *B. stearothermophilus* JY144, and the difference in expression level appeared clearly in the activity-stained gel after PAGE under nondenaturing conditions (Fig. 5A).

For a clear comparison, each of a predetermined amount of cells (30 mg), wild-type and recombinant, was added to the reaction mixture (10 ml) containing 10 mM *rac*-ketoprofen ethyl ester as a substrate. As shown in Fig. 5B, the recombinant enzyme continued to produce the (*R*)-ketoprofen as the reaction proceeded, and a complete conversion was achieved within 25 h, with a strict selectivity to (*R*)-enantiomer (>98%). When the identical conditions were used for the conversion with wild-type cells, the reaction was severely retarded and then stopped within 6–7 h, resulting in conversion of less than 20% of that of recombinant enzyme in the conditions conducted here. The drastic difference, especially in early termination of the reaction, was mainly due to the expression level of the esterase, membrane permeability to the substrate and abundance of proteases in wild-type strain *B. stearothermophilus* JY144.

We report here the screening and identification of a novel strain *B. stearothermophilus* JY144 that expressed an enantioselective esterase to (*R*)-ketoprofen. Further analyses of the esterase gene showed interesting features as a novel family of the esterases uncharacterized to date. To our knowledge, this is a

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Q06174          -----MMKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHG
NP-391242       ----MSMKVVTPKPFTFKGGDKAVLLLHGFTGNTADVRMLGRYLNERGYTCHAPQYEGHG
NP-244421       ------MKLVAPKPFTFEGGTRAVLLLHGFTGTTADVRMLGRYLQEKGYTCHAPLYKGHG
NP-465973       ------MKITPPQPFLFEKGKRAVLLLHGFTGSSADVRILGRFLQENNYTCYAPQYRGHG
JY144           -MSEQYPVLSGAEPFYAENGPVGVLLVHGFTGTPHSMRPLAEAYAKAGYTVCLPRLKGHG
T35504          -----MSVLPGAEPFRHEGGDVGVLLCHGFTGSPQSLRPWARYLAARGLTVSLPLLPGHG
NP-228828       MNFPRCKTVKKSLPIFLEGGNEGVLFIHGYTGSPHDFEYMAKEVNRAGFTVSVPRLPGHG
                         :  . *:  : *  .**: **:**.. ...  ..     . *   *   ***

Q06174          VPPEELVHTGPDDWWQDVMNGYEFLKNKGYEKIAVAGLSLGGVFSLKLGYTVP--IEGIV
NP-391242       VPPEELVHTGPEDWWKNVMDGYEYLKSEGYESIAACGLSLGGVFSLKLGYTVP--IKGIV
NP-244421       VPPEELIQTGPDDWWEDVEDGYQHLKEQGYEEIAVCGLSLGGVFSLKIGYTLP--VKGIV
NP-465973       VSPDLLLKTGPNDWWEDVLEAYDHLKSLGYTEIAVAGLSLGGLFSLKLGFSRP--LKGII
JY144           THYEDMERTTFHDWVASVEEGYGWLKQR-CQTIFVTGLSMGGTLTLYLAEHHPD-ICGIV
T35504          TRWQDMQVTGWQDWYAEVDRELRALRER-CERVFVAGLSMGGALALRLAAKHGDAVSGVV
NP-228828       TCGEDFLTTTARDWLRRAFDAYYDLKAI-CDRVYVVGLSMGGVIALILASQMN--PPKLV
                .  : :  *   **   .      *:      : . ***:** ::* :.         ::

Q06174          TMCAPMYIKSEETMYEGVLEYAREYKKREGKSEEQ----IEQEMEKFKQTPMKTLKALQE
NP-391242       PMCAPMHIKSEEVMYQGVLSYARNYKKFEGKSPEQ----IEEEMKEFEKTPMNTLKALQD
NP-244421       PMCAPMRPKTDDAIYKGVLEYAEEYKRREKKSDEQ----IEEEMERFKSAPQTTLFGLKQ
NP-465973       AMSTPTRMDSSSPIIQGFLDYVRNYKKLEGKTTEQ----IDAEMVAYKDAPMNTIAKLKD
JY144           PINAAVDIPAIAAGMTGGGELPR-YLDSIGSDLKN----PDVKELAYEKTPTASLLQLAR
T35504          VVNPANRMHGVAQHALPVLRHLVPATKGIASDIAK----PLSTELGYDRVPLHSAHSLRA
NP-228828       TLAAATHVFDKRIVLTPILKLFTKKMPCENTEKYEDPDIEYLRKEYWSYNWPKQAAELYK
                 : ..                          .   :          :.         *

Q06174          LIADVRDHLDLIYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQ
NP-391242       LIADVRNNVDMIYSPTFVVQARHDHMINTESANIIYNEVETDDKQLKWYEESGHVITLDK
NP-244421       LIEDVRDHLDHIYAPVFVVQARHDEMIDVESANVIHDTVESDEKSLKWYEDSTHVITLDK
NP-465973       EISGVVAEIDMIYAPIMVVQGEKDDMVDVSGAQLIYDTVESTKKELHWFKESGHVITLDK
JY144           LMAQTKAKLDRIVCPALIFVSDEDHVVPPGNADIIFQGISSTEKEIVRLRNSYHVATLDY
T35504          FFRLADGDLPQVTQPLLLLRSPQDHVVPPADSARILGRVSSTDVTEILLEQSYHVATLDH
NP-228828       LMKLARKSVSKITSATLVVAAKNDNMVPMKAAEFIYNNIRSEKRKLLVFEKSGHVLSNDV
                 :  .   :  :  . ::. . .*.::    :  *   : :        ..* ** : *

Q06174          EKDQLHEDIYAFLESLDW------------------
NP-391242       ERDLVHQDVYEFLEKLDW------------------
NP-244421       EKEQLHEDVYRFLEGLNWSE-----------------
NP-465973       ERKEVNQAILTFLDSLDWQE-----------------
JY144           DQPMIIERSLEFFAKHAGZ-----------------
T35504          DADRIFAESVAFIGRLAPGSVGEPESGLGKEGTAAGG
NP-228828       EKEDVTRAVIEWLKGE--------------------
                :   :      ::
```

Fig. 4. Multiple sequence alignment of the esterase from JY144 with other related enzymes. The alignment includes a full sequence of a carboxyl esterase (Q06174) from *B. stearothermophilis* and probable enzymes from *Streptomyces coelicolor* (T35504), *B. subtilis* (NP-391242), *Thermotoga maritima* (NP-228828), *B. halodurans* (NP-244421) and *Listeria monocytogenes* (NP-465973). Identical and similar amino acid residues among a novel family enzyme are marked by asterisk and point, respectively.

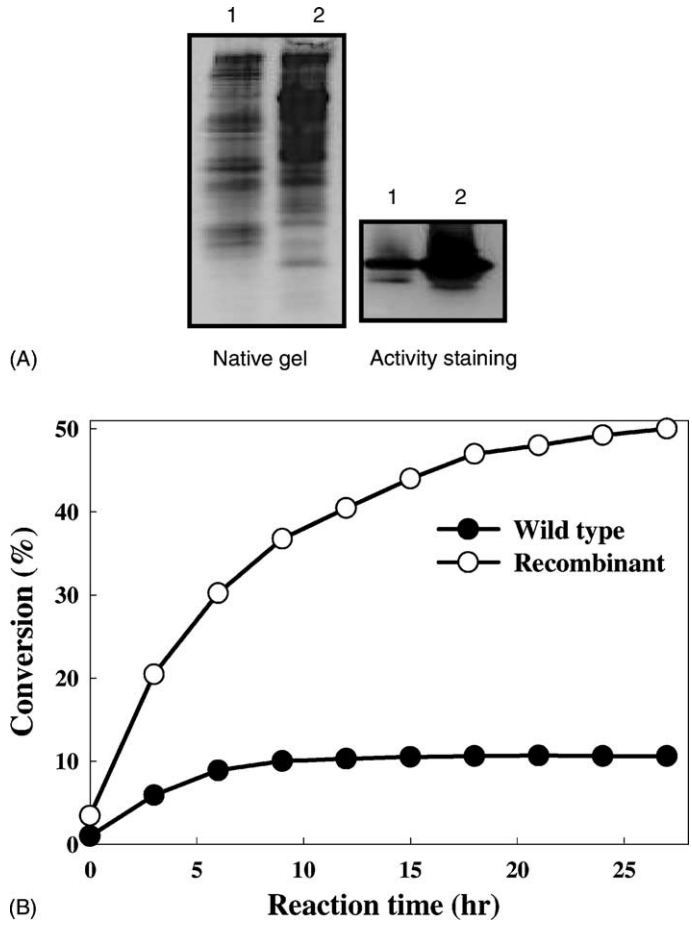

Fig. 5. Activity staining and small-scale conversion with wild-type and recombinant enzymes. (A) Activity staining. Each lane contained 35 μg of proteins. Lane 1: crude extract of JY144 cells; lane 2: crude extract of recombinant *E. coli* cells. (B) Small-scale conversion of (*R*,*S*)-ketoprofen ethyl ester by wild-type and recombinant enzymes. The conversion experiments were conducted according to the conditions described in experimental sections.

first report concerning the gene encoding a stereospecific esterase to ketoprofen, and also providing a recombinant enzyme for commercial production of the optically pure (*R*)-ketoprofen from the corresponding ethyl ester [11]. As for the practical application, however, the expression level and specific activity of the enzyme remained to be further improved, although the enzyme exhibited a great value of enantioselectivity (>99%) and a complete conversion.

Esterases (or lipases) from various sources hydrolyze a broad range of substrates derived from natural or unnatural esters compounds [8,13,19,32,33]. In each case, most of enzymes shared a substrate spectrum and thus had the capability for hydrolyzing a structurally related set of substrates. The esterase from *B. stearothermophilus* JY144 may be able to hydrolyze the substrates unattempted here. With given properties, although the apparent observations that the enzyme acted distinctly on the ester derivative, (*R*,*S*)-ketoprofen ethyl ester, the physiological function and evolutionary relationship between the related enzymes are still unknown [11]. Additional information on these aspects, however, will be obtained intensively from the comparative study and detailed characterizations of related enzymes, most of them are presumed as a novel family.

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