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Identification, molecular cloning and expression of a new esterase from *Pseudomonas* sp. KCTC 10122BP with enantioselectivity towards racemic ketoprofen ethyl ester

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Abstract

A newly isolated gene from *Pseudomonas* sp. KCTC 10122BP, encoding an esterase with enantioselectivity towards racemic ketoprofen (*rac*-ketoprofen) ethyl ester, was cloned in *Escherichia coli* and its nucleotide sequence determined. The deduced amino acid sequence predicted an open reading frame (ORF) encoding a polypeptide of 381 amino acid residues (1143 nucleotides) with a calculated isoelectric point of pH 5.32 and molecular mass of 41,149 Da. The primary structure of the enzyme exhibited a significant level of homology (>31%) with those of related enzymes from various sources and an extreme homology (>81%) with five esterases from the genus *Pseudomonas*. The enzyme was expressed at a high level in an active form in the soluble fraction and purified to homogeneity by a successive chromatographic procedure. The purified enzyme was determined to be a monomer, plus it exhibited a strict selectivity (>99%) and high activity (2360 units/mg-protein) towards (*S*)-ketoprofen ethyl ester.

Keywords: Ketoprofen; Enantioselective; Esterase; *Pseudomonas*; Conversion

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of 2-arylpropionic acids that are widely used for alleviating pain and inflammation associated with tissue injury [1]. Ketoprofen [(*R*,*S*)-2-(3-benzoylphenyl) propionic acid], an in vitro inhibitor of prostaglandin synthesis, is one of the most prevalent anti-inflammatory agents among NSAIDs [2,3]. As with other chiral drugs, the pharmacological activity of ketoprofen is mainly exerted by an enantiomer (mainly *S*-form), although the contaminating enantiomer often has a very poor activity and unexpected side effects in vivo [4]. Currently, ketoprofen is produced in huge quantities by chemical synthesis and sold as a mixture of stereoisomers [5]. Therefore, considerable efforts have been devoted to the enzymatic synthesis of optically pure ketoprofen for pharmacological purposes.

Two kinds of hydrolases (esterases and lipases) with a high enantio- and regio-selectivity have been considered as potential catalysts in the chiral-specific resolution of racemic ketoprofen (*rac*-ketoprofen)

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ethyl ester [6–8]. A lipase from *Candida rugosa* (CRL) has exhibited a relatively high activity towards (*S*)-enantiomers [9,10]. But, the enantioselectivity of the enzyme towards the ethyl ester form of (*S*)-ketoprofen is insufficient when the enzyme is used under general conditions [10]. In an effort to increase the enantioselectivity of CRL, a novel two-step acetone treatment method was attempted and successfully applied [9]. Recently, an esterase from a yeast strain *Trichosporon brassicae* CGMCC 0574 has been identified and shown high enantioselectivity to the ethyl ester form of (*S*)-ketoprofen [11]. Interestingly, two new esterases have been identified as (*R*)-ketoprofen ethyl ester-specific, one from a yeast strain *Citeromyces matriensis* CGMCC 0573 [12] and another from a bacterium *Bacillus stearothermophilus* JY144 [13].

To isolate a novel esterase with a high specificity to the hydrolysis of (*S*)-ketoprofen ethyl ester, the current authors recently conducted a screening experiment and isolated strain KCTC 10122BP (formerly S34 strain) that was taxonomically identified as a *Pseudomonas* sp. [14]. Although the whole cell enzyme of *Pseudomonas* sp. KCTC 10122BP exhibited a strict selectivity towards (*S*)-ketoprofen, the poor conversion rate requires further improvement.

This study describes the cloning, overexpression, and characterization of a new esterase with a high activity and strict stereoselectivity towards the (*S*)-ketoprofen ethyl ester from *Pseudomonas* sp. KCTC 10122BP. It would appear that this is the first report on an enzyme that has the potential for mass production of optically pure (*S*)-ketoprofen from its corresponding racemic ethyl ester.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Pseudomonas sp. KCTC 10122BP, a previously isolated strain with a high selectivity towards (*S*)-ketoprofen ethyl ester, was used as the esterase gene source for the cloning experiment. *E. coli* DH5α and BL-21(DE3) were employed as host strains in the gene manipulation and protein expression, respectively. The plasmids used for the cloning, subcloning, and protein expression were pUC119, pBluescript II KS+ (Stratagene), and pET22b (Novagen), respectively.

Pseudomonas sp. KCTC 10122BP was grown at 30 °C in a nutrient broth (0.3% beef extract, 0.5% peptone) or TS media formulated previously [14]. Wild-type and recombinant *E. coli* cells were subcultured regularly in a Luria–Bertani (LB) medium at 37 °C with an appropriate amount of ampicillin (100 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to the culture media or solid plates when needed. The cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer. A tributyrin agar based on addition of tributyrin (1%, v/v) into the LB medium was prepared for the primary screening of the recombinant clones [14,15].

2.2. Cloning of esterase gene from Pseudomonas sp. KCTC 10122BP

The chromosomal DNA was isolated from *Pseudomonas* sp. KCTC 10122BP using an isolation kit (Promega), partially digested with *Sau*3AI, and then ligated with a *Bam*HI-digested pUC119 vector. The resulting constructs were transformed into the host *E. coli* DH5α by electroporation.

The *E. coli* transformants were spread and grown on agar plates in the presence of X-gal, then the positive clones harboring the plasmid with the inserted DNA were initially screened using a defective α-complementation of β-galactosidase [16]. Next, the positive clones that expressed an esterase and/or lipase were further screened using a 1% (v/v) tributyrin agar plate and the formation of a clear zone around the colony. After incubation at 37 °C for 24 h, those clones exhibiting distinct activity were picked up and re-spread on a fresh plate. The well-isolated colonies were inoculated and cultivated in an LB medium at 37 °C for 24–30 h, then harvested. The resulting pellets were subjected to an enzyme assay using *rac*-ketoprofen ethyl ester as the substrate. Those clones showing a preference for (*S*)-enantiomer with a high conversion yield were finally screened and stored at −20 °C for further analyses.

2.3. DNA sequence determination

For nucleotide sequencing, the DNA fragment cloned within the selected strain harboring the plasmid pUC/EST1 was analyzed with a physical map

using restriction enzymes, and the fragments subcloned into a pBluescript II KS+. The resulting plasmid, pBS/EST14, was used for the sequence analysis as the template. Nested deletion sets of the 2.2 kb insert from pBS/EST14 were constructed from the end, generated by a *Not*I and *Xba*I double-cut of the multicloning site, in a unidirectional mode by digestion with exonuclease III. The derivatives generated with different sizes of inserts were isolated, blunt-ended, and subcloned for sequence analyses. Nucleotide sequencing was performed on both strands using an automatic sequencer (ABI Prism 377, Perkin-Elmer). Double-stranded DNA or PCR-amplified fragments were used as the template with either universal or synthetic primers when needed. The nucleotide sequence was reported to the GenBank and assigned accession number AF380303.

2.4. Construction of expression vector pEESTa

For high-level expression, the esterase gene was PCR-amplified using a *Taq* DNA polymerase-premix kit (QIAGEN) and set of synthetic primers, N-terminal primer: 5′-GGGAATTTCCATATGCAGATTCAGGGACATTACGAGCTTCAATTC-3′ and C-terminal primer: 5′-CCGCTCGAGTTACAGACAAGTGGCTAGTACCCGCGCCAG-3′. The restriction sites, *Nde*I and *Xho*I, were introduced into the N- and C-terminal primers, respectively. The amplified DNA fragment (1.2 kb) was purified and subcloned into the *Nde*I/*Xho*I sites of pET22b(+). The resulting construct was designated pEESTa and confirmed by DNA sequencing.

2.5. High-level expression and purification of enzyme

E. coli BL21 (DE3) (Stratagen) was transformed with pEESTa and the recombinant protein was expressed with various concentrations of IPTG at different temperature for the optimal expression. The recombinant enzyme was purified to apparent homogeneity based on a previous report with slight modifications [17].

2.6. Characterization of esterase from Pseudomonas sp. KCTC 10122BP

The molecular mass and oligomeric structure of the enzyme were determined using an FPLC system with a gel filtration column (Superdex-75, Pharmacia). The flow rate of the mobile phase containing 50 mM Tris–HCl and 150 mM NaCl was 0.5 ml/min. The column was calibrated using native protein markers (Amersham Pharmacia Biotech). A molecular mass standard curve was established by plotting the elution time or volume of the protein markers versus the known molecular masses on semi-log paper. Based on an aliquot of each fraction eluted, the homogeneity and activity were confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and an enzyme assay, respectively.

2.7. Stereo- and substrate-specificity of esterase EST-A

The substrate specificity was analyzed based on the ability to release *p*-nitrophenol from various ester compounds [18]. The purified enzyme was incubated with 5 mM ester derivatives in 3 ml of a Tris–HCl buffer (50 mM, pH 8.5) at 30 °C for 10 min, then the reaction rates were estimated by measuring the increase in the absorbance at 410 nm.

The *rac*-ketoprofen ethyl ester, as a typical substrate for chiral resolution, was prepared according to a general method for esterification, based on a previous report [9]. The hydrolyzing activity on the ketoprofen ethyl ester was determined at 30 °C for 30–60 min with the purified enzyme (1–3 μg) in 3 ml of a reaction mixture containing 50 mM Tris–HCl (pH 8.5), 0.3% Triton X-100, and 5 mM of the substrate. The reaction was stopped by the addition of four volumes of ethanol and the precipitates removed by centrifugation. The resulting solution was then analyzed using high performance liquid chromatography [14]. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of ketoprofen per min under the specified conditions. All the assays were carried out in triplicate and the mean values estimated.

3. Results and discussion

3.1. Cloning of esterase gene from Pseudomonas sp. KCTC 10122BP

The genomic DNA isolated from *Pseudomonas* sp. KCTC 10122BP was partially digested with

*Sau*3AI (or *Mbo*I) and the fragments (2–5 kb) eluted from the agarose gel. The resulting fragments were ligated into the *Bam*HI site of pUC119, and the constructs transformed into *E. coli* DH5α. The resulting transformants were then spread directly on a tributyrin plate or typical LB plate containing X-gal. In the latter case, those colonies showing positive signals were transferred to the selective plate by tooth-picking. A single clone that exhibited a distinct clear zone with no defective growth was obtained out of 3500 independent colonies.

3.2. Physical map and sequence analyses

The plasmid DNA of the clone, screened and confirmed by the above steps, was isolated and then analyzed with restriction endonucleases (Fig. 1). The esterase activity was found to be associated with a 3 kb insert, designated as pUC/EST1. Based on the restriction map, each fragment was subcloned and one carrying a 2.2 kb insert (*Xba*I–*Pst*I fragment) was found to exhibit distinct esterase activity (pUC/EST14). To identify a possible open reading frame (ORF) encoding an esterase, the nucleotide sequence of the 2.2 kb insert from pUC/EST14 was completely determined and scanned on the GenBank database. An analysis of the DNA sequence showed the whole size of the inert to be 2145 bp and revealed two distinct ORFs designating an esterase and probable membrane protein (Fig. 1). One of the open reading frames (ORF1) consisted of 1146 bp, and its deduced amino acid sequence exhibited a significant level of homology to the reported sequences of a subset family of esterases from various sources, yet mainly the genus *Pseudomonas* [19–21]. The amino acid sequence deduced from the other open reading frame (ORF2), an incomplete open reading frame that was truncated at its C-terminus and located downstream from ORF1, exhibited a high homology to probable outer membrane proteins. A similar sequence and genetic organization were also previously reported as probable proteins in the completed genome sequence of *P. aeruginosa* [21].

3.3. Characterization of esterase gene from *Pseudomonas sp.* KCTC 10122BP

The deduced amino acid sequence from the putative esterase of ORF1 revealed an open reading frame of 381 amino acid residues with a calculated molecular mass of 41,149 kDa (GeneBank accession number AF380303). The calculated molecular mass was in good agreement with that determined by SDS-PAGE (data not shown). Similar to previous reports on related gene coding for esterases, the ORF utilized GTG as a start codon rather than ATG [19]. In addition, possible sequences corresponding to typical elements of *Pseudomonas* promoters [22] were also found in the upstream region of the predicted ORF. Finally, a relatively high G + C content (65%) in the ORFs, which is a well-known feature of *Pseudomonas* chromosomes [21], was found in the predicted ORFs.

The BLAST search results showed a close relationship between the KCTC 10122BP esterase and a subset of esterase family enzymes. The best scores (81–90%) were observed with five enzymes, a probable (NP_249738) and four identified esterases (AF228666, JC2091, A44832 and 2006221A) from strains of *Pseudomonas*, when conservative substitu-

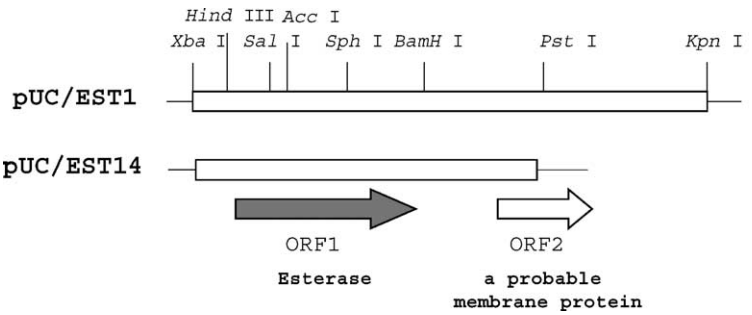

Fig. 1. Physical map and genetic organization of DNA fragment cloned in pUC/EST1. Open arrows indicate the proposed direction and extension of the putative ORFs.

tions were considered. In particular, two enzymes, the esterase EstA from the *Pseudomonas*sp. [20] and a carboxyl esterase from *P. fluorescens* [16], displayed quite a high homology (90%) to KCTC 10122BP esterase. Accordingly, these results strongly suggest that the other five esterases may also be able to hydrolyze *rac*-ketoprofen ethyl ester into (*R*)- or (*S*)-ketoprofen, although such activity has not yet been reported.

3.4. High-level expression and purification

To identify and characterize the enzyme encoded by the predicted gene, *E. coli* BL21 (DE3) cells with pEESTa was induced with 1 mM IPTG at 37 °C for 3 h. As shown in Fig. 2, cell extracts of the recombinant *E. coli* showed a distinct protein band (>5%), corresponding to the size of the expected esterase. However, the soluble fraction was estimated to be about <35% of the expressed enzyme, while the major portion was detected in the insoluble fraction (lane 4 of Fig. 2).

In an attempt to increase the soluble fraction of the enzyme, the enzyme was expressed using various concentrations of IPTG at different temperatures. As a result, a significant increase in the expression level in the soluble fraction was observed when the enzyme was induced at 20 °C with a lower concentration of IPTG (0. 25–0.4 mM) (lane 6 in Fig. 2). The esterase appeared as a major band in the soluble fraction, and its expression level reached about 5–8% of the total cellular proteins. Approximately, 75% of the esterase was soluble, plus, under these conditions, the enzyme exhibited a 110–130-fold increase in activity towards the (*S*)-ketoprofen ethyl ester, compared to that of *Pseudomonas* sp. KCTC 10122BP. The other factors, such as the host cells, induction time, and ester derivatives added as an inducer, did not significantly improve the solubility of the enzyme (data not shown).

To investigate the biochemical properties of the esterase in detail, purification experiment was conducted at 4 °C. Using induced cells under predetermined conditions, the purification was carried out using a cell extract from 200 ml of a culture broth. As expected, the esterase activity was mainly detected in the supernatant fraction, with a portion of the protein (<20%) remaining as an insoluble aggregate in the cell pellet. The crude extract was concentrated and subjected to a SDS-PAGE analysis. As shown in Fig. 3, a distinct band appeared on the SDS-PAGE (lane 2). After clarification by filtration, the crude cell extract was loaded onto an ion exchange chromatography column. The active fractions eluted with NaCl ranging from 0.25 to 0.35 M were collected, concentrated, and

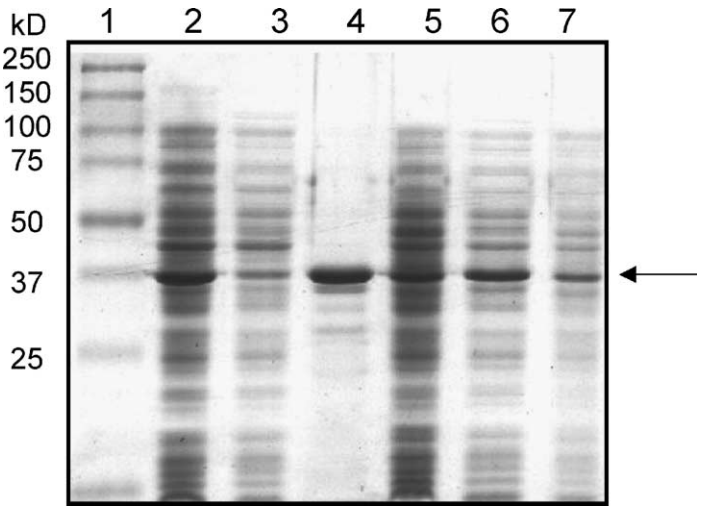

Fig. 2. SDS-PAGE analysis of high-level expression of esterase in *E. coli* under different conditions. The protein samples were loaded onto a 12% polyacrylamide gel under denaturing conditions, then the resolved bands were stained with Coomassie brilliant blue. Lane 1, molecular weight markers; lane 2, whole cell extract (37 °C); lane 3, soluble fraction (37 °C); lane 4, insoluble fraction (37 °C); lane 5, whole cell extract (20 °C); lane 6, soluble fraction (20 °C); lane 7, insoluble fraction (20 °C). Arrow indicates the esterase expressed.

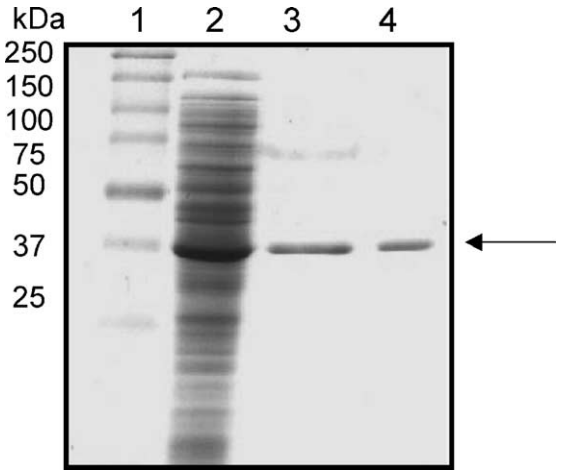

Fig. 3. SDS-PAGE analysis of esterase purified from successive steps. The protein samples were prepared from *E. coli* cells grown and induced at 20 °C. Lane 1, molecular weight markers; lane 2, whole cell extract; lane 3, eluted fractions from ion exchange chromatography; lane 4, eluted fraction from gel filtration chromatography. Arrow indicates the esterase purified.

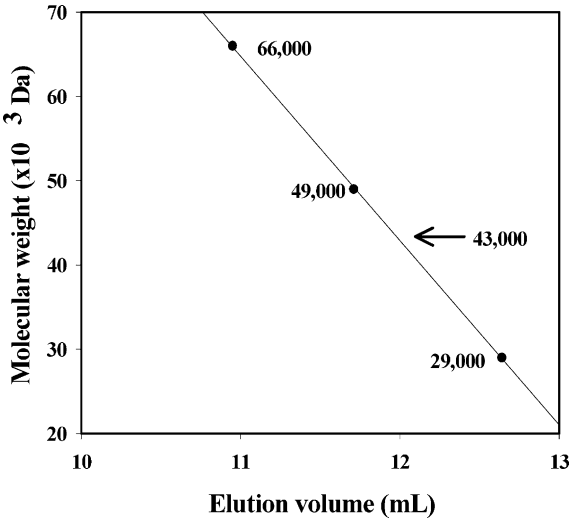

Fig. 4. Gel-filtration chromatogram of esterase from *Pseudomonas* sp. KCTC 10122BP. The molecular mass of 43,000 Da was estimated from the elution profiles of standard protein markers.

further resolved using a Sephacryl S-200-HR gel filtration column. After this simple two-step procedure, the esterase was purified to homogeneity judged from an SDS-PAGE gel (Fig. 3). The purification steps and recovery yields are summarized in Table 1.

3.5. Characterization of recombinant esterase

The molecular mass and oligomeric structure of the enzyme were first determined by gel filtration column chromatography (Superdex-75). The apparent molecular mass was estimated to be 40–45 kDa from the gel filtration chromatography, which was similar to that calculated from its primary structure. This indicates that the quaternary structure of the enzyme was monomeric (Fig. 4). No higher molecular weight aggregates or oligomeric structures resulting from non-specific interactions with the monomer appeared under the current analytical conditions. Native gel electrophoresis and activity staining under non-denaturing conditions also supported these results (data not shown).

Concerning the substrate specificity, Table 2 shows the specific activities of the purified enzyme towards

Table 1
Purification procedures and yields of esterase from recombinant *E. coli*

Step	Total protein (mg)	Specific activity[a] (units/mg-protein)	Purification fold
Crude extract	25.9	250	1.0
Ion exchange	5.3	950	3.8
Gel-filtration	1.2	2300	9.1

[a] The activity was determined using the fractionated enzymes under standard assay conditions (50 mM Tris–HCl (pH 8.0), 0.3% Triton X-100).

Table 2
Activity and substrate specificity of esterase from *Pseudomonas* sp. KCTC 10122BP

Substrate	Specific activity[a] (units/mg-protein)	Relative activity (%)
p-Nitrophenyl acetate	14700	38
p-Nitrophenyl butyrate	38500	100
p-Nitrophenyl caprylate	>5	>1
p-Nitrophenyl palmitate	N.D.[b]	–
Ketoprofen ethyl ester	2360	6

[a] The enzyme activity was determined by adding the purified enzyme to a reaction mixture containing a 4–5 mM substrate and 3 ml of a 50 mM Tris–HCl (pH 8.0) buffer. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of product per min. The reaction products were analyzed either by HPLC or using a spectrophotometer.

[b] N.D.: not detected under specified conditions.

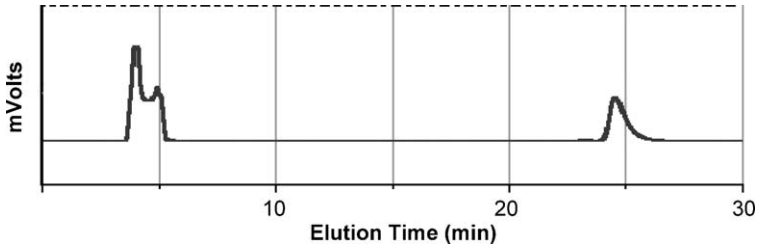

Fig. 5. HPLC chromatogram of chirally resolved (*S*)-ketoprofen. The reaction products resulting from the activity of the purified enzyme were analyzed on a chiral column and identified with authentic samples.

various ester derivatives, including *rac*-ketoprofen ethyl ester. The enzyme was not able to catalyze the two esters, *p*-nitrophenyl palmitate and *p*-nitrophenyl caprylate. Under standard assay conditions (pH 8.5 and 30 °C), the esterase exhibited the highest activity towards *p*-nitrophenyl butyrate (38,500 unit/mg-protein). The specific activity was calculated to be as high as 2360 units/mg-protein for *rac*-ketoprofen ethyl ester, with an absolute stereoselectivity towards (*S*)-enantiomer (Fig. 5), making the enzyme to be one of the favorable catalysts for the production of (*S*)-ketopofen.

Acknowledgements

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