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Enhanced thermostability and tolerance of high substrate concentration of an esterase by directed evolution

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Abstract

A newly isolated enantioselective esterase from *Pseudomonas fluorescens* KCTC 1767, which is currently considered as a biocatalyst for the production of a commercially valuable (*S*)-ketoprofen, has revealed a low structural and thermal stability. In order to enhance the stability, directed evolution was attempted on this enantioselective esterase by successive steps of an error prone and staggered extension PCR. After the second round of evolution, the best mutant 6–52 with enhanced thermal stability was selected and analyzed. DNA sequence analyses of 6–52 revealed that the three amino acid residues (L120P, I208V, and T249A) were changed and the mutation L120P was presumed as a structurally important residue due to its presence in all positive variants. The purified mutant 6–52, when incubated at 50 and 55 °C for 2 h, remained its activity over 30 and 10%, respectively, whereas there were no detectable activities in wild-type enzyme. The analysis of 6–52 in the presence of 15% ethanol showed 1.8-fold increase in the activity, compared to that of wild-type enzyme. The K_m and V_{max} values of 6–52 were estimated to be slightly increased, leading to 1.2-fold-higher the catalytic efficacy k_{cat}/K_m than that of wild-type enzyme. Additionally, the mutant 6–52 was more resistant to high substrate concentrations than that of wild-type enzyme.

Keywords: Ketoprofen; Esterase; Directed evolution; Stabilization

1. Introduction

Enzyme-mediated chiral resolutions of racemic compounds can regiospecifically work on substrate (*s*) in exclusive manner under mild conditions [1,2]. The employment of enzymes, therefore, is considered to be the most promising alternative for organic synthesis, as the demands for new chemicals and the resulting waste disposals increase. Although the potentials of enzyme are fully evaluated, low stability and activity often limit general use of enzymes as industrial catalysts [3]. In this context, tailor-made or engineering strategy for improving the properties of enzymes has attracted and become a major goal of recent researches [4,5]. Currently, two parallel approaches, a rational knowledge-based design and irrational random mutagenesis of enzymes, have been employed independently or corporately in certain cases due to concerted effects [6]. As one of random approaches for protein engineering, directed evolution techniques have proven to be an efficient route for improving the properties of enzymes even without information of structure–function relationship, thus yielding a number of promising results [7,8]. In this process, diverse pools of mutant library are generated by typical in vitro random mutagenesis and recombination of genes. Especially, family-driven homologous recombination is considered as the most effective means for generating mutant diversity by accelerating in vitro evolution [9].

Ester–hydrolases, including esterase and lipase, can catalyze the stereo-specific hydrolysis of various ester bonds and, thus, were employed industrially in the production of an enantiomer from its corresponding ethyl ester forms [1,2,10]. As a practical case, chiral resolution of *rac*-ketoprofen ethyl ester to (*S*)- or (*R*)-ketoprofen is recently attempted by using an esterase [11–13]. Of these,

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the (*S*)-ketoprofen [(*S*)-2-(3-benzoylphenyl) propionic acid] is one of the most prevalent anti-inflammatory agents, thus, attracting as a target for enzyme-mediated chiral resolution [14]. The other enantiomer (*R*)-ketoprofen, preventing periodontal disease as a toothpaste supplement, is also converted chirally from amide [15] or ester form of ketoprofen [16].

Ketoprofen ethyl ester-hydrolyzing enzymes, mainly esterases, have been screened from various sources and evaluated their potential for the chiral resolution of *rac*-ketoprofen ethyl ester [14–18]. Among them, the esterase from *Pseudomonas fluorescens* KCTC1767 was reported that the enzyme had a high activity to the ethyl ester form and also known to be well expressed [18]. However, the enzyme was found to have a low structural and thermal stability even when the enzyme was incubated with a substrate, which was considered to be a limiting factor in the process development [17]. Here, we describe an in vitro evolution of the esterase from KCTC 1767 for improvement of thermostability. Error-prone PCR was used to generate a mutant library, and resulting mutant pool was further recombined by staggered extension PCR (StEP) process [19]. After these steps, the best mutant showing a distinct improvement was selected and characterized in detail.

2. Materials and methods

2.1. Enzymes and reagents

The *rac*-ketoprofen ethyl ester was prepared according to the previous report [20]. Fast Blue RR, r*ac*-ketoprofen, α-naphthyl acetate, ethoxyethanol, (*R*)- and (*S*)-ketoprofen were purchased from Sigma. Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were purchased from Promega. All other reagents used were of analytical grade.

2.2. Mutant library construction

The esterase gene of *P. fluorescens* KCTC1767 was used as the template for the construction of mutant library [17]. Under low stringent conditions, an error-prone PCR was conducted with a set of primers, N-terminal ESTF and C-terminal primer ESTR [17]. The reaction mixture composed of 0.2 mM dNTP, 30 pmol primer, 2.5 ng template and 7–10 mM $MgCl_2$, and PCR conditions were as follows: 1 cycle: 94 °C, 10 min; 45 cycles: 94 °C, 1 min, 48 °C, 1 min, 72 °C, 1 min; 1 cycle: 72 °C, 10 min. The resulting genes were purified and digested with *Bam*HI and *Pst*I, and then subcloned into an expression vector pQE30. The transformed *Escherichia coli* JM109 with these constructs was spread on Luria–Bertani (LB) plate containing ampicillin (75 μg/ml) and grown overnight at 37 °C for activity screening.

Plasmids were purified from positive clones in the first round of random mutagenesis and then used for the next round of in vitro evolution by StEP process [19]. The StEP process, carried out with the reaction mixture containing 0.2 mM dNTP, 0.4 pmol primer, 0.15 pmol template and 2 mM $MgCl_2$, was as follows: 1 cycle: 94 °C, 5 min; 95 cycles: 94 °C, 30 s, 50 °C, 20 s; 1 cycle: 72 °C, 10 min. *Taq* DNA polymerase was used for recombination, and *pfu* DNA polymerase was added when needed [19]. The resulting genes were subcloned into the same vector, as used in the first round of library construction.

2.3. Screening of positive clones and DNA sequencing

The positive clones expressing variant enzymes with enhanced stability were screened by following steps (Fig. 1). After incubation of transformed cells on LB plates for 24 h, recombinants were replicated onto new plates and further incubated at 37 °C for 12–14 h, followed by heat treatment step at 45–60 °C for 2 h. The resulting clones were activity-stained by using an overlaid soft agar (0.6%) containing 15 mg/ml of Fast Blue RR and 45 mg/ml of α-naphthyl acetate [21]. The positive clones rapidly developed a deep brown color around the colony, as reported previously [16].

The primarily screened clones were picked with sterile toothpicks from master plates and placed into 96-well plates containing 0.2 ml of LB medium, followed by incubation at 37 °C for 24 h. An aliquot (0.1 ml) of cells was harvested and transferred into new 96-well plates containing 0.1 ml of 50 mM Tris–HCl buffer (pH 8.0). After heat treatment for 2 h, the remained activity was detected by activity staining [18]. The resulting candidates were finally subjected to activity analyses using heat-treated crude extracts and *rac*-ketoprofen ethyl ester. The recombinant cells of the first and the second round were thermally treated at 45–50 and 55–60 °C, respectively, for 2 h in a dry oven.

DNA sequence analyses were performed on both strands using a synthetic or universal primer. The nucleotide and amino acid sequences were aligned by hierarchical clustering of the individual sequence using Clustal W program [22].

2.4. Enzyme purification and oligomeric structure analysis

E. coli cells were grown in 250 ml of LB broth and induced with 0.2 mM IPTG for 2.5 h when the $OD_{600\,nm}$ reached about 0.45. The collected cells by centrifugation were suspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole, and disrupted by ultrasonification. After centrifugation at 12,000 × *g* for 30 min, the supernatant was treated with 0.2% protamine sulfate for further clarification. Both soluble and insoluble fractions were subjected to enzyme assay and SDS–PAGE (12%). The soluble fraction was loaded onto an affinity resin, Ni–NTA (Qiagen), pre-equilibrated with the cell lysis buffer. The column was completely washed with the same buffer and then eluted with a buffer containing 250 mM imidazole.

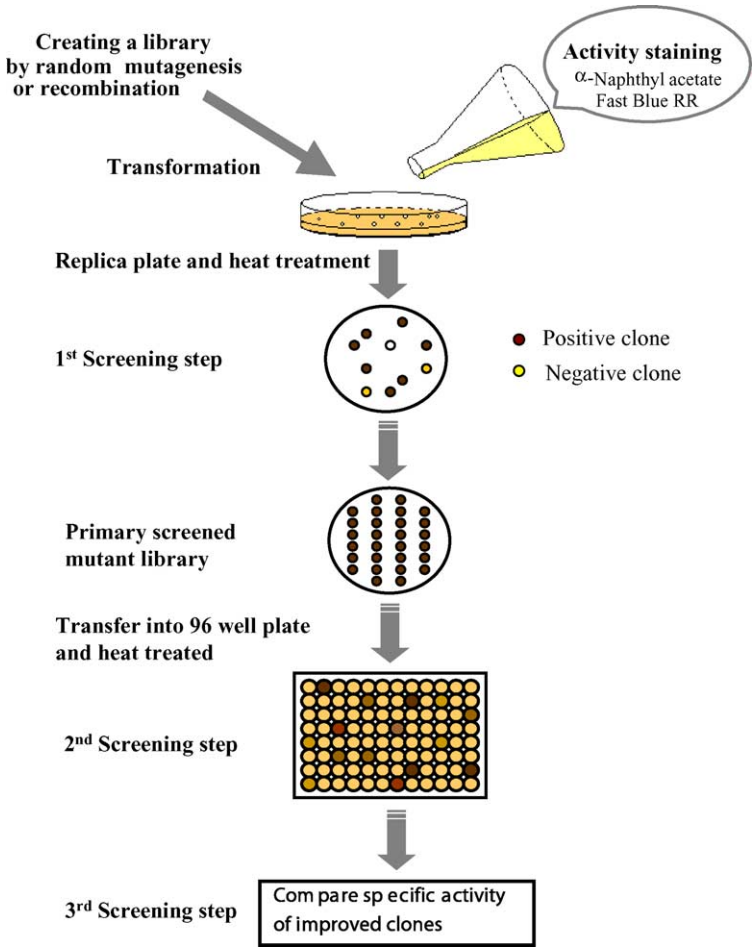

Fig. 1. Typical procedure of the consecutive reaction steps for the screening of potential clones with enhanced stability and retained activity of enzyme from mutant pools.

The changes in the oligomeric structure and folding state of variant enzymes were determined on a FPLC system with a Superdex-75 gel filtration column. The flow rate and mobile phase used were 0.5 ml/min and 20 mM Tris–HCl (pH 8.0, 150 mM NaCl), respectively. A molecular mass standard curve was established by plotting the elution profiles of protein markers (Sigma) versus the known molecular masses on a semi-log paper. Aliquots of each eluted fraction were subjected to SDS–PAGE and enzyme assay.

2.5. Temperature, pH, and additive effects

To determine the thermal stability, the purified enzymes were preheated at 25–60 °C for 2 h in 0.1 M Tris–HCl buffer (pH 8.0), and the residual activity was analyzed under standard assay conditions. The pH stability was also determined by measuring the residual activity after the incubation of purified enzymes at 30 °C for 2 h using following buffers: citrate (pH 5.0–6.0), phosphate (pH 6.0–7.5), Tris–HCl (pH 7.5–9.0) and glycine–NaOH (pH 9.0–10.0). The effects of surfactants and solvents on the enzyme activity were also determined according to the previous report [17].

2.6. Substrate spectrum and enzyme assay

Substrate specificity was analyzed by the releasing activity of *p*-nitrophenol from various ester compounds [17,23]. The purified enzymes (2 μg) were incubated with an ester derivative (10 mM) in 3 ml of 50 mM Tris–HCl buffer (pH 8.5) at 35 °C for 10 min, and the specific activity was calculated by measuring the increase in the absorbance at 405 nm. The hydrolyzing activity on ketoprofen ethyl ester (1–100 mM) was determined at 35 °C for 30 min in 0.5 ml of reaction mixture containing 50 mM Tris–HCl (pH 8.5) and 0.15% Triton X-100. The reaction was stopped by adding four volumes of ethanol, and then analyzed using high performance liquid chromatography (waters) in terms of conversion yield and enantioselectivity [17,18]. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of ketoprofen per minute under specified conditions.

2.7. Activity staining on native gel and analyses

For the activity staining on native gel, protein samples were mixed with 0.2 volume of native sample buffer and

resolved on a 12% PAGE. After gel electrophoresis, half of the separating gel was washed twice with 20 ml of 50 mM Tris–HCl buffer and then soaked into the same buffer containing 4 mg α-naphthyl acetate dissolved in 0.5 ml ethoxyethanol. The activity band was developed by dropping Fast Blue RR (2 mg/ml) solution.

3. Results and discussion

Since the utilization of esterases as catalysts for the chiral resolution of *rac*-ketoprofen ethyl ester has recently been suggested by some reports [13,14,16,17], screening and practical application of related enzymes become a recent subject. In this context, enriched strains from various sources had been previously tested as possible candidates. A lot of enriched strains in the medium supplemented with or without ketoprofen ethyl ester as an inducer showed distinct activities, strongly suggested a broad distribution of related enzymes in nature. Among them, a potential esterase from *P. fluorescens* KCTC1767 was identified by a systematic approach [18]. However, further reactions in a practical scale were partly unsuccessful due to the low stability of enzyme, requiring an in vitro evolution for practical use. To screen a potential variant, mutant pool was subjected to successive screening steps at each round of evolution (Fig. 1). First, the variants that showed a distinct stability after heat treatment were selected. With these clones, further screening was conducted to isolate the variants that retained, or rather increased, the activity of wild-type enzyme. These screening steps were necessary due to the incompatibility between the high stability and high activity at moderate, or high temperature, although a few reports suggested compatibility between two properties [24,25].

3.1. Directed evolution of an esterase from KCTC 1767

From the mutant pool of the first round of random mutagenesis, about 2500 clones were subjected to the screening of variant enzymes with enhanced stability. As a control, the wild-type enzyme showed an activity to be about <3% of the initial activity when heat-treated at 45–50 °C, thus, gave no color change by activity staining within 10 min. This condition was suitable for the screening of variant enzymes with improved stability. From the first round of mutant pool, 23 positive clones were primarily screened and further confirmed with the crude extracts of induced cells, by comparing the residual activity after thermal inactivation. Further comparison of specific activity was made to select the stabilized variants without a loss of activity, yielding five variants with enhanced stability and retained activity. To create more potential pool of mutant library, five clones were used for the second round of evolution by StEP process. Screening of about 3500 clones, after heat inactivation step at 55 °C on solid agar plate, resulted in 35 positive clones with more improved stability than that of wild-type enzyme. Further analyses of thermostability and specific activity by using crude extracts showed the clone 6–52 as the best variant that revealed the highest stability and conversion yield (Table 1).

Table 1
Conversion yield and enantioselectivity of mutant clones finally considered

Strain	Before heat treatment		After heat treatment	
	Conversion (%)	ee_p (%)[a]	Conversion (%)	ee_p (%)
Wild type	28.9	100	0.2	100
6–52	43.4	94.1	12.8	100
6–53	31.6	95.9	0.6	100
6–41	25.9	89.1	3.6	71.3
7–3	20.7	100	0.7	100
7–20	38.1	95.5	2.3	100
7–34	39.3	95.3	0.6	100
7–35	41.6	96.1	1.2	100
7–47	26.2	100	3.1	100
8–23	36.0	95.7	0.9	100
8–36	35.6	95.8	2.9	100
8–37	42.2	95.7	4.3	100
8–51	33.2	96.3	0.5	100
9–3	24.3	100	2.9	100
9–14	28.4	96.2	3.7	100
9–17	33.1	96.1	2.8	100
9–26	43.6	95.3	3.8	75.0
10–4	38.5	96.7	0.6	100
10–11	36.2	100	2.8	100
10–12	25.0	100	2.4	100

[a] Enatiomeric excess (ee_p):

$$ee_p\,(\%) = \frac{[(S)\text{-ketoprofen}] - [(R)\text{-ketoprofen}]}{[(S)\text{-ketoprofen}] + [(R)\text{-ketoprofen}]} \times 100$$

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Using 6–52 as the template gene, mutant pool of the third round was generated, but further enhancement was not achieved. Although a possibility that may generate a stabilized variant by alternative approach, such as saturation mutagenesis, was remained, the mutant 6–52 was selected for characterization. During the screening procedure, the variants with altered enantioselectivity or high tendency to aggregation were intentionally excluded, although the stability was increased.

3.2. Amino acid substitutions in the evolved enzyme 6–52

Sequencing of the gene encoding 6–52 revealed five point mutations. Among them, three mutations changed the amino acid residues (L120P, I208V, and T249A) and two mutations were silent. The mutation causing a frame shift was not found. From the analyses of mutated residues, we observed an interesting mutation, L120P, found in all of the variants (clones 6–52, 8–37, 9–26, and 10–11) sequenced. The mutation T249A resulted in a replacement of hydrophilic residue with a hydrophobic one. Therefore, the two substitutions, L120P and T249A, could provide a possibility of increasing structural rigidity by more favorable packing. The mutation I208V was thought to be conservative. The consensus sequences, H–G and G–X–S–X–G, of the related esterases

and lipases [16,18,21,26] were not mutated. Currently, the specific assignments of the changed residues are difficult because no structural data are available. However, it seems that the cooperative effect exerted by these mutations confers favorable protein folding, thereby leading to improved stability of 6–52. In order to further evaluate the contribution of each residue to enhanced stability, site-directed mutagenesis is under progress. There is no report on the amino acid substitution identified in this work for improving the thermostability.

3.3. Expression of the evolved enzyme 6–52

As for a clear comparison, the mutant 6–52 was induced and expressed under identical conditions of wild-type enzyme. When induced with 0.2 mM IPTG at 30 °C for 150 min, the apparent activity of 6–52 toward *rac*-ketoprofen ethyl ester was estimated to be slightly higher than that of the wild-type enzyme. As a distinct difference between two enzymes, the expression of 6–52 was dominant (>80%) in soluble fraction. A considerable portion (30–45%) of the wild type enzyme, however, was detected in insoluble fraction. The expression levels of both enzymes were estimated to be about 17–25% of total cell protein. From these results, it is reasonable to assume that a high activity of 6–52 after heat inactivation step can be mainly attributed to improved stability, or folding ability, rather than increased catalytic activity and expression level. This assumption was further confirmed by using the purified enzymes to apparent homogeneity.

3.4. Characterization of the selected variant 6–52

As a determinant of in vitro evolution, thermostability of the purified 6–52 was determined and compared with that of the wild-type enzyme. As shown in Fig. 2, 6–52 retained about 30% of the initial activity after heat treatment at 50 °C for 2 h, whereas no considerable activity of the wild-type enzyme revealed. Moreover, the variant 6–52 resulted in a detectable activity (>10%) even when heated at 55 °C for 2 h. Along with the improved thermal stability, 6–52 was also more stabilized at a range of alkaline pH (8.5–12) than wild-type enzyme (Fig. 2B). The purified wild-type enzyme lost about 20–30% of its activity under the same conditions. In the optimal temperature, 6–52 showed an activity maximum at 35 °C, which was about 5 °C higher than that of wild type enzyme [17]. No distinct difference in pH optimum between two enzymes was also observed, and both enzymes showed maximum activities at pH 9.0–10.0.

From the eluted fractions of gel filtration column chromatography, 6–52 was found to be eluted with an apparent molecular mass between 40 and 45 kDa. No higher molecular aggregates or oligomeric structures were found even in a high concentration of purified enzymes. Therefore, the molecular mass and oligomeric structure of 6–52 were identical to those of the wild-type enzyme [17].

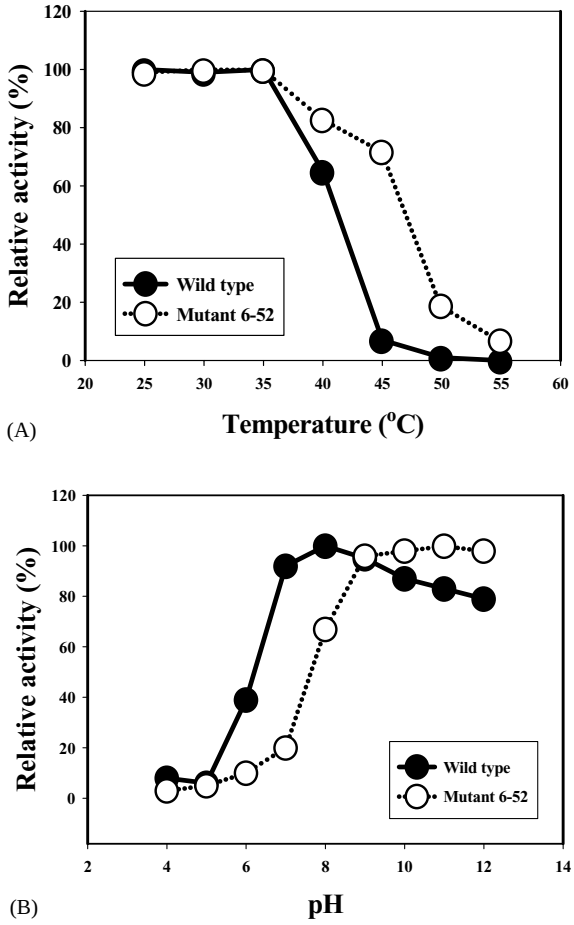

Fig. 2. Effects of temperature (A) and pH (B) on the stability of evolved mutant 6–52. To determine the stability, purified enzymes were preincubated for 2 h at the indicated pH and temperature, and the remaining activity was determined under standard assay conditions.

The wild-type enzyme had shown a strong dependency on a nonionic detergent Triton X-100, as observed in related esterases and other lipases [14,17,20]. The mutant 6–52 also resulted in an explosive increase in the activity when treated with nonionic detergents (Table 2). The addition of either Tween 40 (1%) or Triton X-100 (1%) led to drastic increases in the activity of each case, but further enhancements were

Table 2
Effects of nonionic detergents on the activity of the evolved mutant 6–52

	Relative activity[a] (%)	ee_p (%)
Control	100	98.6
Tween 20	560	96.8
Tween 40	780	96.1
Tween 60	620	94.1
Triton X-15	610	94.4
Triton X-45	1010	95.6
Triton X-100	1200	97.5
Triton X-165	850	95.8

[a] Relative activity was determined in the reaction mixture containing 1 μg enzyme, 5 mM *rac*-ketoprofen ethyl ester, and 1% surfactant under standard assay conditions

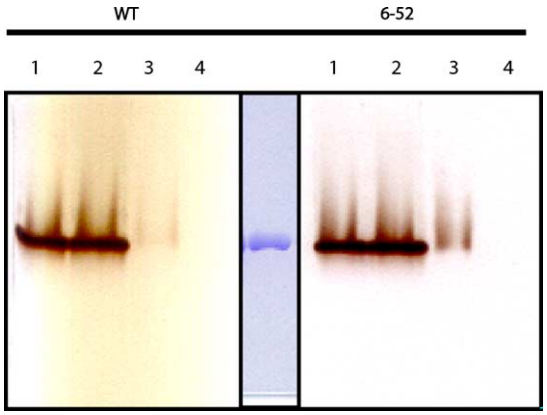

Fig. 3. Comparison of SDS susceptibility between the evolved 6–52 and wild-type enzyme. The purified protein (1 μg) was activity-stained on a 12% gel after electrophoresis with 0.001% (lane 1), 0.01% (lane 2), 0.05% (lane 3), and 0.1% (lane 4) SDS. The resolved protein on the identical gel was also Coomassie blue-stained as a control.

not achieved by varying incubation time and detergent concentration. A significant difference in enantioselectivity was not found in the presence of these detergents. As observed in wild type enzyme (Fig. 3), an ionic detergent SDS also strictly inhibited the activity of 6–52 even at a quite low concentration (<0.05%). When ethanol (15%) was used as an additive, 1.8-fold increase in the conversion yield was obtained, providing a possibility that the increased thermostability also resulted in enhanced resistance to an organic solvent.

Fig. 4A shows the specific activities of the purified 6–52 toward some typical substrates including *rac*-ketoprofen ethyl ester. As shown in wild-type enzyme, 6–52 revealed a relatively high activity to *p*-nitrophenyl with short acyl chain, whereas no detectable activity for a long chain acyl compound (C16) was observed. Therefore, this enzyme displayed a similar substrate spectrum of the wild-type enzyme [17]. To *rac*-ketoprofen ethyl ester, 6–52 showed a 1.5-fold-higher activity (4.12 U/mg protein) than that of wild-type enzyme at a substrate concentration of 10 mM. The K_m and V_{max} values were calculated to be 1.5 mM and 6.2 μmol/mg protein/min and for *rac*-ketoprofen ethyl ester, respectively, resulting in a 1.2-fold increase in the catalytic efficacy k_{cat}/K_m when compared with the wild-type enzyme [17]. During kinetic studies, the enzyme consistently retained the strict selectivity to (*S*)-enantiomer.

3.5. Enhanced substrate tolerance and conversion yield of the evolved 6–52

It has been generally known that an enzyme activity is frequently inhibited by the presence of its own substrate at relatively high concentrations. When used the identical units of enzymes for clear comparison, the activity of wild type enzyme was considerably affected by the target substrate *rac*-ketoprofen ethyl ester. As shown in Fig. 4B, a distinct decrease (>30%) in the activity of wild-type enzyme was observed in the presence of 5 mM substrate, and about 60–63% of initial activity was observed at substrates ranging of 10–100 mM. As a control experiment, the identical units of enzymes showed a close activity and conversion profiles at a relatively low substrate concentration of <5 mM. These results indicated that in the substrate range of 10–100 mM, the conversion yields of 6–52 was about 1.5–1.7-fold higher than those of wild type enzyme. Furthermore, the mutant enzyme showed about 2–2.5-fold more conversion than wild-type enzyme when the reaction was performed at 40 °C. The product (*S*)-ketoprofen showed a negligible effect on the enzyme activity under the same conditions.

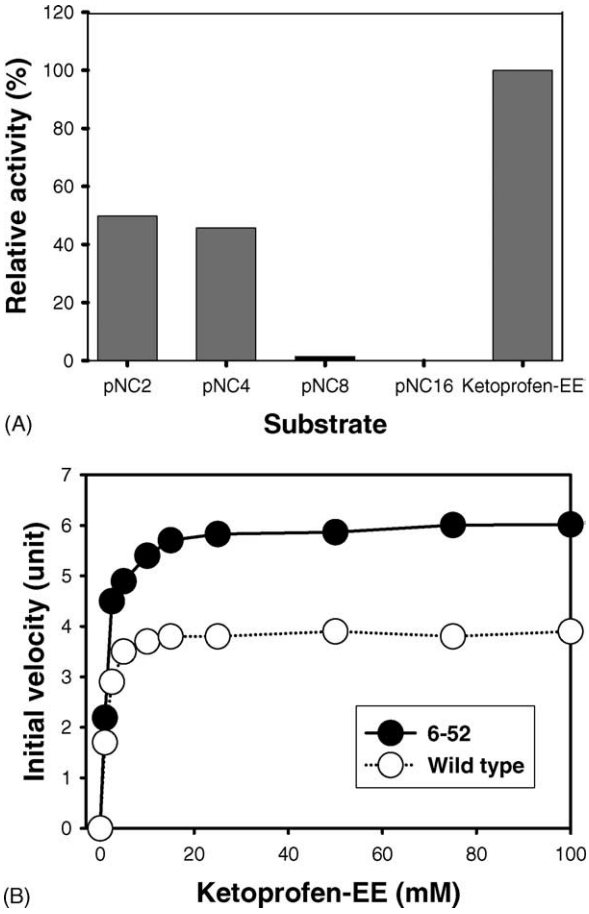

Fig. 4. Substrate specificity and tolerance of high substrate concentrations of 6–52. The substrate specificity was determined with the purified enzymes and each substrate (10 mM) in 3 ml of 50 mM Tris–HCl buffer (pH 8.5), by using either HPLC or spectrophotometer (A). The initial velocity of the mutant and wild type enzyme was analyzed in 0.5 ml of reaction mixture containing the identical unit of each enzyme and indicated amount of substrates (B).

To further evaluate the potential of the evolved enzyme for practical use, small-scale conversions were attempted and compared with that of wild-type enzyme. The reactions were carried out with relatively low protein concentrations of the purified enzymes, in order to evaluate the enhanced protein stability and also tolerance to high substrate concentration of

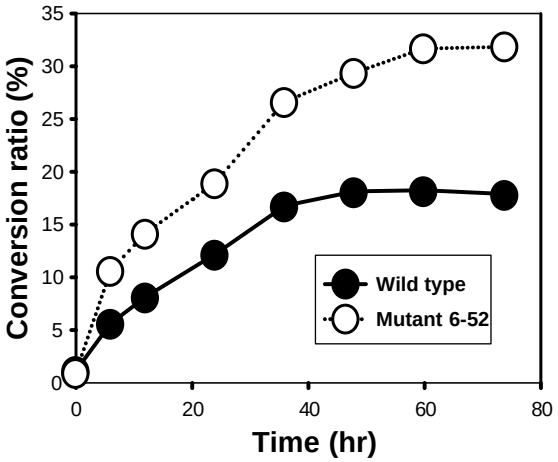

Fig. 5. The small-scale conversion experiment with the purified enzymes (9.5 μg) at 35 °C and 180 rpm. The conversion profiles were shown as converted percents of (*S*)-ketoprofen from 100 mM *rac*-ketoprofen ethyl ester.

the evolved enzyme. A predetermined amount of the purified 6–52 (9.5 μg) was added to the reaction mixture (3 ml) containing 100 mM *rac*-ketoprofen ethyl ester, and the resulting conversion profiles, as well as the enantiomeric excess, were analyzed (Fig. 5). Production of (*S*)-ketoprofen continued to increase as the reaction proceeded, and about 32% of the initial substrate was converted to (*S*)-ketoprofen within 60 h. A negligible amount of (*R*)-ketoprofen (<0.05%) was detected in this reaction. In the case of wild-type enzyme, the reaction nearly stopped in 35 h, and the product concentration was determined to be about 53% of that of 6–52. From these results, it is reasonable to assume that a better performance of 6–52 than wild-type enzyme can be attributed to improved stability and tolerance of high substrate concentration, although direct and precise calculations of each effect on improved performance were difficult.

There are a few reports for mutations that lead to the simultaneous improvement of multiple properties of enzymes, such as activity, stability, and substrate tolerance [24,25]. The reason is because that an improvement of any one property is likely to be obtained at the cost of another one, unless multiple constraints are imposed simultaneously [27]. However, in the case of 6–52, only three mutations (L120P, I208V, and T249A) resulted in an enhanced thermostability and substrate tolerance, as well as a slight increase in the catalytic efficacy. These results might be resulted from the successive step, described in the screening procedure of experimental section. Hence it is plausible that the improvement of multiple properties is obtainable more than expected [27].

In conclusion, this study shows that the thermostability and substrate tolerance of an esterase can be improved by directed evolution, without the loss of the catalytic efficacy. Obviously, small-scale conversion using the evolved enzyme shows higher performance than wild-type enzyme, suggesting that the variant may be effectively used as a novel biocatalyst for the production of (*S*)-ketoprofen. The sequence information of 6–52 also provides a basis for further improvement of related family enzymes.

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