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Enzymatic resolution of methyl DL-β-acetylthioisobutyrate and DL-β-acetylthioisobutyramide using a stereoselective esterase from *Pseudomonas putida* IFO12996

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Abstract

Esterase (*Pp*EST) from *Pseudomonas putida* IFO12996 catalyzes the stereoselective hydrolysis of methyl DL-β-acetylthioisobutyrate (DL-MATI) and DL-β-acetylthioisobutyramide (DL-ATIA) to give D-β-acetylthioisobutyric acid (DAT). DAT is a key intermediate for the synthesis of a series of angiotensin converting enzyme inhibitors. To use enzyme for the DAT production, the *Pp*EST gene of *P. putida* IFO12996 was cloned and expressed in *Escherichia coli*. *Pp*EST with a molecular weight of 33 kDa could hydrolyze DL-MATI and DL-ATIA to give DAT with enantiometric excess value (e.e. value) about 97% and enantioselectivity value (*E*-value) >150, respectively. The kinetic constants of *Pp*EST for DL-MATI and DL-ATIA were examined and they showed that DL-ATIA was a poorer substrate than DL-MATI for *Pp*EST. However, DL-ATIA was 20-fold more soluble in water than DL-MATI, it was more stable than DL-MATI and it did not show substrate inhibition of the *Pp*EST up to 780 mM. This result suggested that *Pp*EST is an esterase but with amidase activity, which can kinetically resolve DL-ATIA to yield DAT and DL-ATIA is a better choice than DL-MATI for industrial production of DAT by the enzymatic resolution method.

Keywords: D-β-Acetylthioisobutyric acid; Captopril; Enzymatic resolution

1. Introduction

Enzymes and whole-cell biocatalysts with unique regio- and stereo-selectivity are suited for the manufacture of optically pure stereoisomers [1]. The demand for optically pure compounds has been increased significantly in pharmaceutical and chemical industries, and there were over 300 biocatalytic processes up to date that have been implemented in industry [2]. D-β-Acetylthioisobutyric acid (DAT) also known as *S*-(−)-3-acetylthio-2-methylpropionic acid is an optically active intermediate required for the synthesis of a series of angiotensin converting enzyme inhibitors, such as captopril and alacepril which are used for the treatment of hypertension and congestive heart failure. With respect to the preparation of DAT, it can be acquired from crystal optical resolution [3] and enzymatic optical resolution [4–7]. However, the crystal optical resolution was time consuming and required expensive agents. The enzymatic optical resolution has gained more attention recently. Gu et al. used lipase to hydrolyze DL-ester to produce D-acid that having 80% enantiomeric excess value (e.e. value) [4]. Bianchi and Cesti also used lipase to hydrolyze DL-MATI and produce D-MATI with an e.e. value of 88% [5]. Sakimae et al. reported the enzymatic hydrolysis of DL-MATI by using an esterase from *Pseudomonas putida* MR-2068 to give DAT with e.e. value of 98.5% [6]. Honda et al. reported that 3,4-dihydrocoumarin hydrolase was able to hydrolyze DL-MATI to produce DAT with high optical purity (>99.9% e.e.) [7].

To be successfully implemented in industrial process, both enzyme and substrate need to have unique properties: the enzymes need to have high stereo-selectivity toward its substrate, can be over expressed in recombinant form, and have high thermal and pH stability. The substrate needs to be kinetically good substrate for the enzymes, water soluble and relatively stable under enzymatic reaction condition. In this report, we have cloned and over expressed the *Pp*EST gene from *P. putida* IFO12996, examined its thermal and pH stability, and

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characterized its kinetic resolution activity toward two substrates, DL-MATI and DL-ATIA, which have different water solubility and stability.

2. Materials and methods

2.1. Chemical and enzymes

Methyl methacrylate (MMA) was obtained from Janssen (Geel, Belgium). Thioacetic acid was purchased from TCI (Tokyo, Japan). Methacrylamide (MAA) was obtained from Acros (Geel, Belgium). All other reagents used in this work were of analytical grade and are commercially available. A unit of esterase activity was defined as using 1 mg enzyme to produce 1 mM DAT per minute at 37 °C and pH 7.0.

2.2. Strains, plasmids and growth media

P. putida IFO12996 was from Institute for Fermentation, Osaka. *Escherichia coli* JM109 and *E. coli* BL21 (DE3) were used for gene cloning and expression, respectively. Microorganisms were cultured on Luria–Bertani (LB) medium at 30 °C for *P. putida* IFO12996 and at 37 °C for *E. coli*. In selective medium, 100 μg/ml of ampicillin was used. pET21d (Invitrogen) was used as cloning and expression vector.

2.3. PCR amplification and nucleotide sequencing of the PpEST gene from P. putida

To amplify the fragment of DNA encoding *Pp*EST from the total genomic DNA of *P. putida* IFO12996 by PCR, two primers flanking the easterase gene sequence of *P. putida* MR2068 [8] were used. Total genomic DNA was isolated from IFO12996 by using the DNeasy Tissue Kit (Qiagen). The primer sets included a 5′-end primer; 5′-GCGAGATCATGAGCTATGTA-ACCACGAA GGAC-3′ and a 3′-end primer; 5′-ACTGCC-GAATTCACATCAGCTACGGATAA ACGCCAGCAAATC-3′ with added restriction *Bsp*HI and *Eco*RI sites (underlined), respectively. The PCR reaction mixture contained 100 ng of genomic DNA, 10 pmol each of the two primers, 100 μM of dNTP, 1.2 mM Mg^{2+} and 0.5 units of *Pfu* polymerase in 25 μl volume. The reaction was run for 30 cycles of 94 °C (30 s), 50 °C (30 s) and 72 °C (90 s). The PCR products were digested with *Bsp*HI and *Eco*RI, purified by agarose gel electrophoresis and ligated into an ampicilin resistant vector pET21d. The nucleotide sequence of the insert was determined by the dideoxy chain termination method using T7 primers. The recombinant plasmid was designated pET-*Pp*EST.

2.4. Expression and purification of P. putida esterase

The recombinant plasmid pET-*Pp*EST was transformed into *E. coli* BL21 (DE3) competent cells. A single colony was picked up and grown in LB medium containing 100 μg/ml ampicillin. The overnight culture was diluted 1:100 with LB medium containing 100 μg/ml ampicillin and allowed to grow until A600 reached 0.3. IPTG was added at final concentration of 0.1 mM and the cells were harvested 3 h. Cells were collected from culture by centrifugation at 10,800 × *g* at 4 °C for 20 min. Cell pellets were suspended with 20 ml Tris–HCl buffer (0.5 M, pH 7.0) per gram of cell pellet and disrupted by using the Microfluidizer M-110Y (Microfluid Corp., MA, USA) at 4 °C. The disrupted cell mixture was centrifuged at 12,000 × *g* for 20 min at 4 °C and the supernatant was collected and precipitated with ammonium sulfate at final concentration of 3 M. The precipitant was collected by centrifugation at 12,000 × *g* for 30 min at 4 °C and then resuspended in 5 ml of Tris–HCl buffer (50 mM, pH 7.0) per 100 mg of precipitant. The resuspended solution was dialyzed overnight against 2 l of Tris–HCl buffer (20 mM, pH 8.0).

The dialyzed sample was clarified by centrifugation at 12,000 × *g* for 20 min and then by filtration through nylon 0.22 μm membrane. After filtration, the sample was loaded onto a FPLC system (Amersham Pharmacia Biotech) with an ion exchange column (Resource Q-6) that had been previously equilibrated with 20 mM Tris–HCl buffer, pH 8.0 (buffer A) at a flow rate of 3 ml/min. The adsorbed proteins were eluted by a linear gradient with buffer B (containing 20 mM Tris–HCl, pH 8.0 and 0.5 M NaCl) while 3-ml fractions were collected. The enzyme activity of each fraction was detected as acid-production by pH indicator.

2.5. SDS-PAGE of P. putida esterase

The molecular weight of *Pp*EST was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out in gels (Bio-Rad) containing 10% acrylamide gel. Protein standards (Amersham Pharmacia Biotech) used for the estimation of molecular masses were phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie blue. Enzyme concentration was measured by BCA protein assay reagent (Pierce).

2.6. Synthesis of DL-MATI from MMA

DL-MATI was synthesized according to a previous published procedure [6]. Briefly, it was prepared by allowing 22 g (0.22 mol) of MMA to react with 25 g (0.33 mol) of thioacetic acid at 95 °C for 6 h, and was purified by distillation under reduced pressure. The reaction mixture was distilled at 78–81 °C to give 37 g (yield 95.5%) of a distillate as pure DL-MATI. The structure of DL-MATI was analyzed by ^{1}H NMR spectrum from Bruker AC-200, FT NMR (200 MHz) in $CDCl_3$ with TMS as an internal reference. The chemical identification of DL-MATI was analyzed by ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 1.23 (3H, d, CH_3), 2.30 (3H, s, CH_3COS), 2.69 (1H, m, CH), 3.0–3.2 (2H, m, SCH_2), 3.67 (3H, s, $COOCH_3$).

2.7. Synthesis of DL-ATIA from MAA

17.2 g (0.2 mol) MAA was dissolved in 100 ml *n*-propyl alcohol and then added 30.4 g (0.4 mol) thioacetic acid and 1 ml

(10 mmol) piperidine. The mixture reacted at room temperature for 4 h. Then the reaction mixture was added with 150 ml, 0.5 M Tris–HCl buffer (pH 7) and adjusted pH to 1 with concentrated H_2SO_4. The mixture was extracted with 100 ml hexane three times and the most of unreacted thioacetic acid was extracted to the hexane layer, then the aqueous solution was adjusted pH to 7 with 10N NaOH and extracted with 100 ml ethyl acetate three times and the DL-amide was extracted to the ethyl acetate layer. Crude DL-amide was obtained followed by condensation of the ethyl acetate extract and pure DL-amide was prepared by crystallization and gave 20.1 g (yield 62.4%). The chemical identification of DL-ATIA was analyzed by ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 1.21 (3 H, d, CH_3), 2.32 (3 H, s, CH_3COS), 2.49 (1 H, m, CH), 2.9–3.1 (2 H, m, SCH_2).

2.8. Measurement of the solubility of DL-MATI and DL-ATIA

Saturated solution of DL-MATI and DL-ATIA were prepared by dissolving each compound in 0.5 M Tris–HCl buffer (pH 7.0) at 37 °C for 30 min. The excess amount of solutes were removed by centrifugation at 10,800 × *g* for 5 min and clear supernatants were injected into HPLC system to quantify the concentration of each sample by comparing to a linear calibration curve of DL-MATI and DL-ATIA, respectively.

2.9. Stability assay of DL-MATI and DL-ATIA

Ten milligrams of DL-MATI or DL-ATIA was dissolved in 1 ml Tris–HCl buffer (50 mM) with three different pH (pH 7–9) and incubated at 67 °C. The concentration of hydrolyzed product from DL-MATI or DL-ATIA in the reaction mixture at different time points were determined in a HPLC system (Shimadzu LC-10AT) with a reverse phase C-18 column (Hypersil BDS).

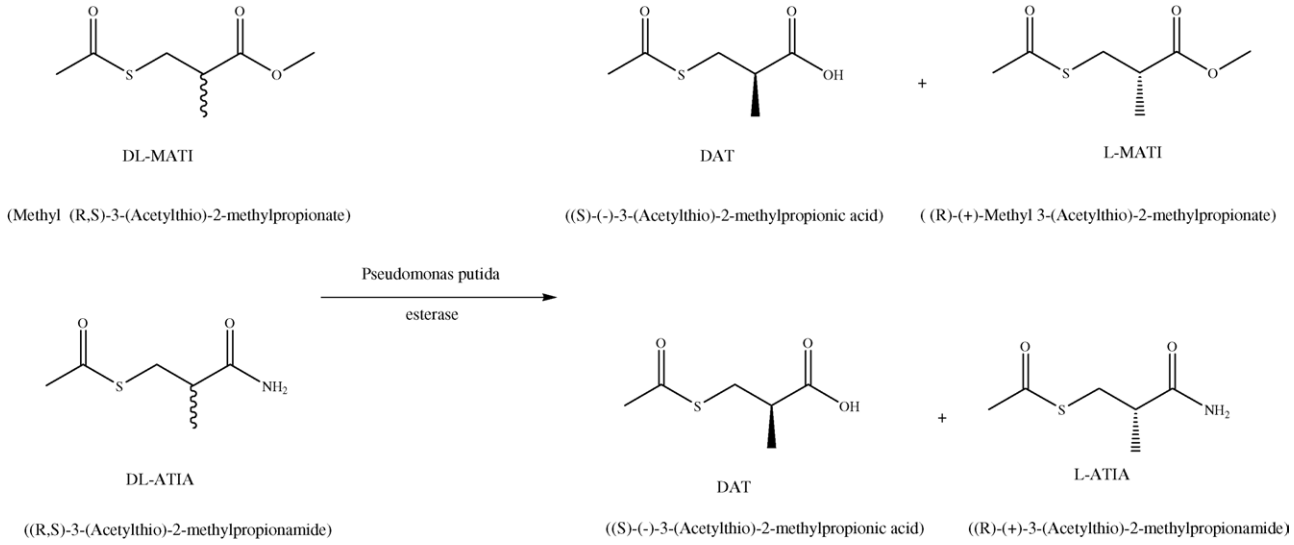
2.10. Enzyme activity assay and DAT purification from DL-MATI and DL-ATIA

Two grams (11 mmol) of DL-MATI and 370 μg of pure *Pp*EST were mixed with 100 ml of 0.5 M Tris–HCl buffer (pH 7.0), followed by reaction at 37 °C for 2 h (Scheme 1). The pH of the reaction mixtures was kept at 7.0 by adding 0.1N NaOH solution by a pH stat (Suntex pH controller PC-310). The concentration of hydrolyzed product from DL-ester in the reaction mixture were determined in a HPLC system (Shimadzu LC-10AT) with a reverse phase C-18 column (Hypersil BDS). Injected samples were eluted with isocratic 50% solvent A (25 mM phosphate buffer, pH 6.5) and solvent B (acetonitrile) and monitored at UV 229 nm. The concentration of eluted sample was quantified by comparing to a linear calibration curve for each sample.

After the reaction, unreacted DL-ester and L-ester in the solution was extracted with 50 ml ethyl acetate three times. The pH of the aqueous layer of the resultant extract was adjusted with diluted sulfuric acid to 2.0. The mixture was then extracted with 50 ml ethyl acetate three times to give a DAT extract. Anhydrous $MgSO_4$ was added to the extract. After dehydration, the solvent was vaporized to give crude DAT and then distilled under reduced pressure to give pure DAT. The optical rotation of the resulting sample was analyzed by a polarimeter (JAS.CO DIP-370). The DAT from DL-ATIA was also purified by the same procedure as it from DL-MATI. The optical rotation of DAT was used to calculate the enantiometric excess value (e.e. value) and enantioselectivity value (*E*-value) according to previously described method [12]. The chemical identification of DAT was analyzed by ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 1.28 (3H, d, CH_3), 2.33 (3H, s, CH_3COS), 2.70 (1H, m, CH), 3.0–3.2 (2H, m, SCH_2).

2.11. Substrate inhibition

Degree of enzyme inhibition by substrates was analyzed by measuring the initial rates under various substrate



Scheme 1. Esterase-catalyzed optical resolution of DL-MATI and DL-ATIA.

concentrations. Reactions were performed by adding 1.5 μg of *Pp*EST into 1 ml of reaction buffer (50 mM Tris–HCl, pH 7.0) containing 0–780 mM DL-ATIA. After incubation at 37 °C for 10 min, the reaction was stopped by adding equal volume of acetonitrile. The concentration of DAT was analyzed by HPLC as described above.

2.12. Kinetics of the enzyme reactions

The kinetic studies of DL-MATI was performed by adding 2.125 μg *Pp*EST in 500 μl of Tris–HCl buffer (0.5 M, pH 7.0) containing 40 mM DL-MATI and the mixtures were incubated at 37 °C for 80 min. The concentrations of DL-MATI/L-MATI and DAT at different time intervals were determined by HPLC system as described above. The kinetic constants (K_m and K_{cat}) were obtained by fitting experiment data with the Microsoft Office program that performs non-linear regression based on the least-square method [9]. The kinetic parameters for DL-ATIA were also determined by adding 170.0 μg *Pp*EST in final volumes of 1000 μl of Tris–HCl buffer containing 780 mM of DL-ATIA and the mixtures were incubated at 37 °C for 250 min.

2.13. Effect of pH on enzymatic conversion

The reaction mixtures (1 ml) containing 15 μg *Pp*EST, 40 mM DL-MATI and buffered solution with different pH (3.0–11.0) were incubated at 37 °C for 5 min and the concentration of DAT was determined as described above.

2.14. Effect of temperature on enzymatic conversion

The reaction mixtures (1 ml) containing 15 μg *Pp*EST and 40 mM DL-MATI were incubated in 0.5 M Tris–HCl buffer (pH 7.0) at various temperature of 17, 27, 37, 47, 57, 67 and 77 °C for 5 min and the concentration of DAT was determined as described above.

2.15. pH stability on enzymatic conversion

The pH stability of the purified enzyme was measured by incubating the enzyme in a series of buffer (pH 3.0–11.0) at 4 °C for 12 h. Each enzyme solutions were then adjusted to pH 7.0 and the remaining activities of the enzymes were determined by incubating with 40 mM DL-MATI at 37 °C for 5 min. The concentration of DAT was determined as described above.

2.16. Thermal stability on enzymatic conversion

The thermal stability measurement for *Pp*EST was done by incubating *Pp*EST solution at temperature of 57, 67, 72 and 77 °C. Every 15 min, 10 μl (7.5 μg) enzyme solution was removed and added in 1 ml Tris–HCl buffer (0.5 M, pH 7.0) containing 40 mM DL-MATI at 37 °C for 5 min. The remaining enzyme activity was determined by measuring DAT as described above.

3. Results and discussion

3.1. Nucleotide sequence and deduced amino acid sequence of PpEST gene

The nucleotide sequence analysis of the PCR amplified product (854 bp) from *P. putida* IFO12996 revealed the sequence of *Pp*EST gene coding for a protein of 276 amino acid residues, and the deduced amino acid sequence exhibited high similarity to esterase from *P. putida* MR-2068 (96% identity) [8] and 3,4-dihydrocoumarin hydrolase from *Acinetobacter calcoaceticus* F46 (73% identity) [10] (Fig. 1). The catalytic triad of typical serine hydrolases, i.e. Gly-X-Ser-X-Gly, aspartate (residue 226 of IFO12996) and histidine (residue 256 of IFO12996) was conserved in these enzymes. The esterase from *P. putida* MR-2068 and the 3,4-dihydrocoumarin hydrolase from *A. calcoaceticus* F46 are able to hydrolyze linear methyl ester and recognize the configuration at the two-position [7,11].

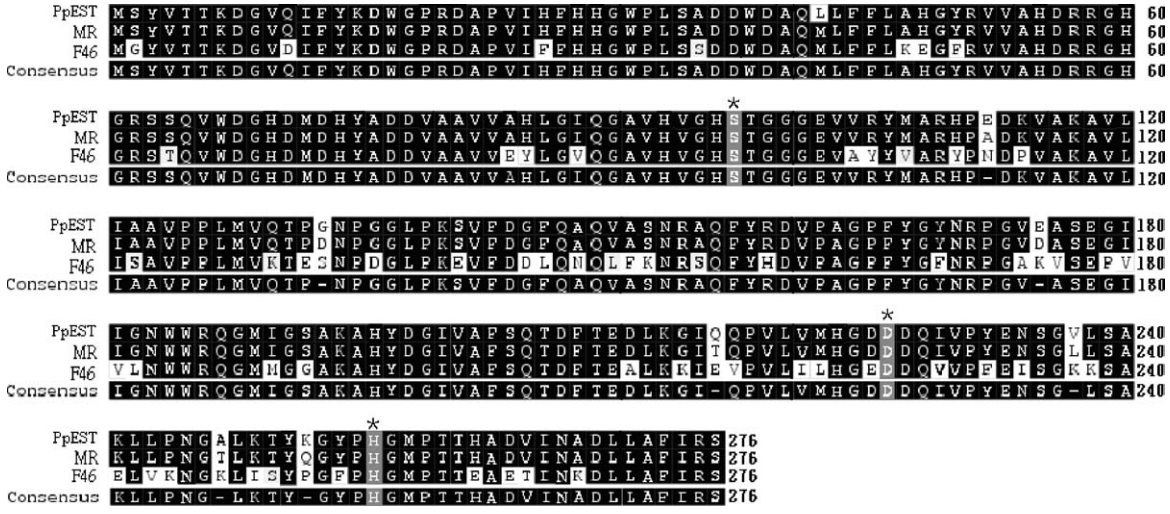


Fig. 1. Alignment of esterase sequences from *Pseudomonas putida* MR-2068 (MR) and IFO12996 (*Pp*EST), and 3,4-dihydrocoumarin hydrolase sequence from *Acinetobacter calcoaceticus* F46 (F46). Ser97, Asp227 and His256 of *Pp*EST residues in the catalytic triad are denoted by an asterisk.

Table 1
Purification of recombinant esterase *E. coli*

Steps	Volume (ml)	Total protein (mg)	Total activity unit (U)	Specific activity (U/mg)	Fold	Yield (%)
Cell-free extract	80	240	8301	35	1.00	100
3 M $(NH_4)_2SO_4$ precipitation	20	114	5231	46	1.33	63
Resource Q 6 ml	30	7.5	3114	415	12.00	38

3.2. Expression and purification of esterase

A cell-free extract of *E. coli* BL21 (DE3) transformed with pET-*Pp*EST exhibited specific DL-MATI hydrolyzing activity of 35 U/mg. A purification procedure involved ammonium sulfate precipitation and ion-exchange chromatography was established to obtain homogeneous esterase for enzyme activity analysis. Specific activity of the esterase during each stage of purification is summarized in Table 1. The molecular weight and purity of *Pp*EST was examined in a SDS-PAGE stained with Coomassie blue (Fig. 2). A major band at 33 kDa was detected in recombinant *E. coli* before and after purification of *Pp*EST, and its mobility did not affect by the presence or absence of reducing agent. This result indicated *Pp*EST with a protein subunit of 33 kDa.

3.3. Measurement of the solubility of DL-MATI and DL-ATIA

Two different racemic compounds, DL-MATI and DL-ATIA, were synthesized as substrates for esterase. The maximum solubility of DL-MATI and DL-ATIA in 0.5 M Tris–HCl buffer (pH 7.0) at 37 °C was 40 and 780 mM, respectively. Since the major difference between DL-MATI and DL-ATIA is in the methyl ester and the amide functional group, it is reasonably expected that DL-ATIA has greater solubility in aqueous solution than DL-MATI does. Esterase is a water-soluble enzyme and it prefers catalyzing reaction in aqueous solution, the higher solubility of DL-ATIA in aqueous solution made it more available for esterase hydrolysis and resulted higher yield of DAT in the final reaction mixtures (Fig. 3). The design of DL-ATIA to make it more water soluble and more accessible to the enzyme was based on the substrate engineering concept reported previously [15].

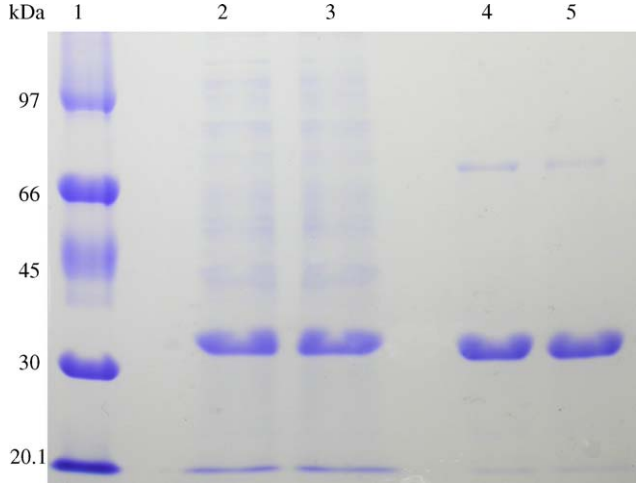


Fig. 2. SDS-PAGE analysis of the recombinant *Pp*EST from *E. coli*. Lane 1: Molecular weight markers. Lane 2: *E. coli* lysate with β-mercaptoethanol. Lane 3: *E. coli* lysate without β-mercaptoethanol treatment. Lane 4: Purified esterase with β-mercaptoethanol. Lane 5: Purified *Pp*EST without β-mercaptoethanol treatment.

3.4. Stability of DL-MATI and DL-ATIA

If the ester or amide bond of DL-MATI or DL-ATIA is hydrolyzed chemically, it will cause the decrease of the optical purity of DAT. Therefore, it is important to know the stability of DL-MATI or DL-ATIA under various pH and temperature. The stability of DL-MATI and DL-ATIA was examined by incubating them in various pH solution and at 67 °C for 120 min. The data in Fig. 3 shows that the hydrolysis of DL-MATI was increased in higher pH and it was significantly higher than that of DL-ATIA under the same pH. This result indicated that DL-ATIA was more stable than DL-MATI.

3.5. Hydrolytic activity toward DL-MATI and DL-ATIA

The hydrolytic activities of *Pp*EST toward two different substrates, DL-MATI and DL-ATIA, were detected by monitoring the concentration of DAT in a reverse phase HPLC system (Fig. 4). The time course of DAT formation from hydrolysis of DL-MATI and DL-ATIA is shown in Fig. 5. After 30 and 180 min reaction, 49.5% of added DL-MATI and 49.0% of added DL-ATIA was converted to DAT with final yield of 45.4 and 42.4%, respectively. The enantiometric excess value (e.e. value) and enantioselectivity value (*E*-value) of DAT from DL-MATI was 97% and >150 while it was also 97% (e.e.) and >150 from DL-ATIA.

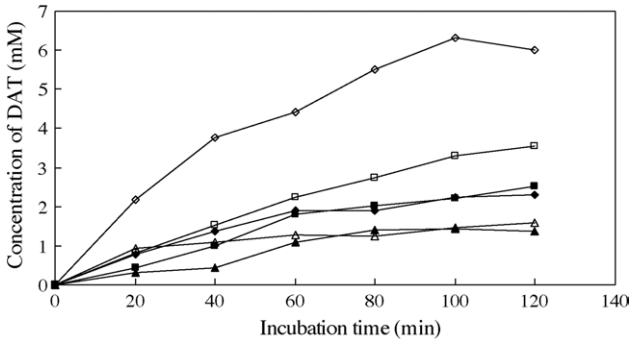


Fig. 3. Substrate stability of DL-MATI (hollow) and DL-ATIA (solid) at 67 °C and different pH. Triangle: pH 7.0; square: pH 8.0; rhombus: pH 9.0.

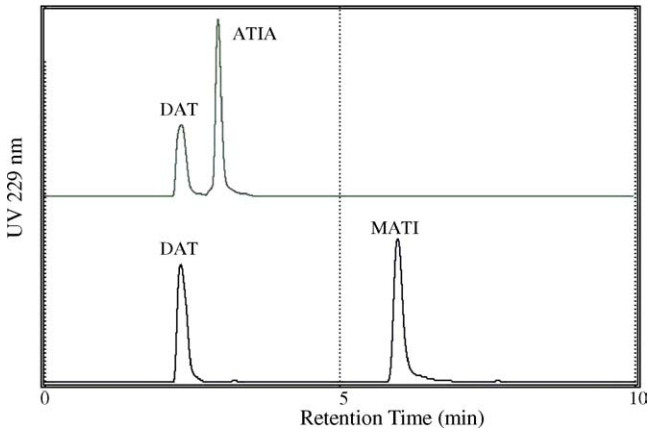


Fig. 4. Typical HPLC chromatogram of the hydrolytic reaction of DL-MATI and DL-ATIA.

3.6. Catalytic property and substrate specificity

The kinetic constants (K_m and K_{cat}) were obtained by fitting experimental data with the Microsoft Office program that performs non-linear regression based on the least-square method [9]. The K_m, K_{cat} and K_{cat}/K_m values of *Pp*EST for DL-MATI were 34 mM, 786 s^{-1} and 23 mM^{-1} s^{-1} and for DL-ATIA was 614 mM, 55 s^{-1} and 0.09 mM^{-1} s^{-1}, respectively. This data indicated that *Pp*EST hydrolyzed DL-MATI 255-fold more efficient than it did DL-ATIA. However, the aqueous solubility of DL-ATIA was 20-fold higher than that of DL-MATI. The effect of substrate concentration on esterase activity was also examined. As shown in Fig. 6, the concentration DL-ATIA up to 780 mM did not show substrate inhibition of the esterase activity, which suggested the DL-ATIA could be a better choice for industrial process of production of DAT.

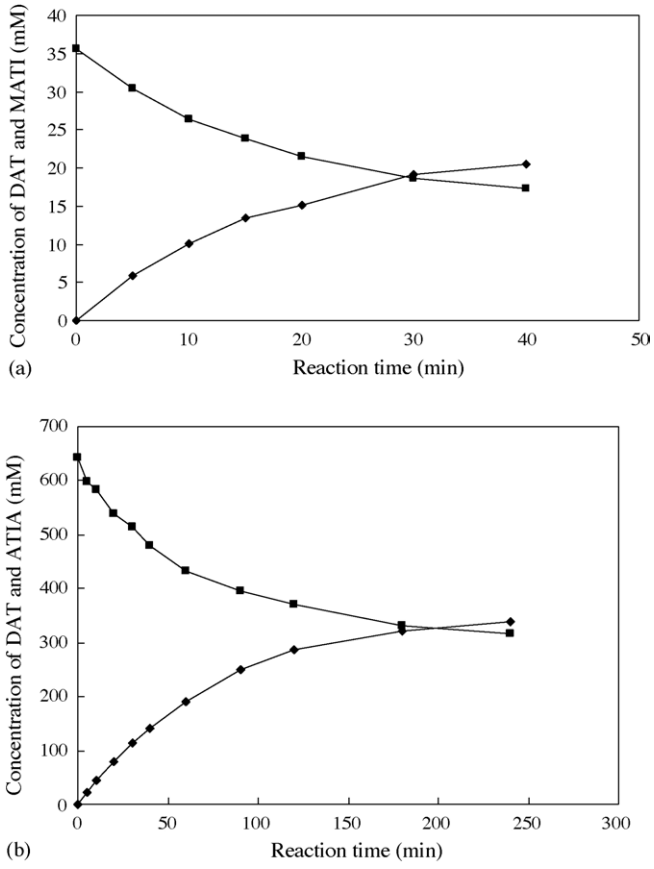


Fig. 5. Asymmetric hydrolysis of DL-MATI and DL-ATIA with *Pp*EST. (a) Course of DAT production from DL-MATI: DL-MATI (■-) and DAT (♦); (b) course of DAT production from DL-ATIA: DL-ATIA (■-) and DAT (♦).

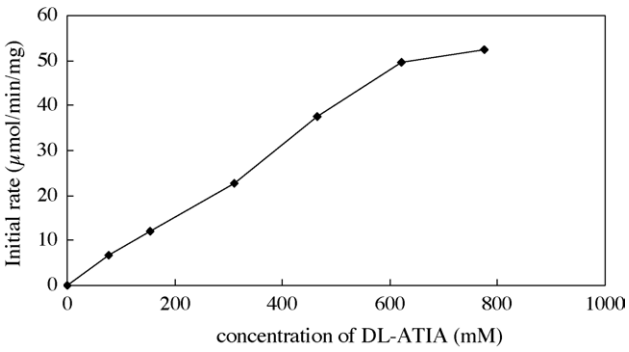


Fig. 6. Effect of substrate concentration on the esterase activity. The initial rate of hydrolysis was plotted against concentration of DL-ATIA.

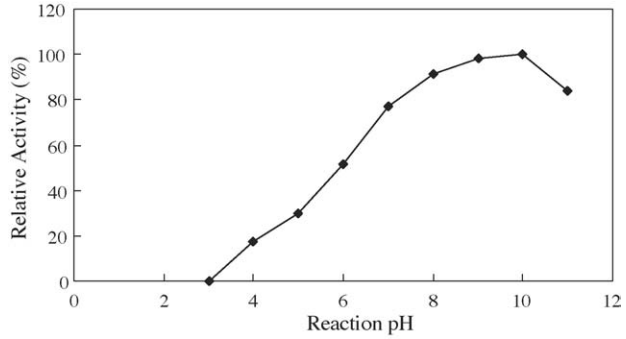


Fig. 7. Effect of pH on enzymatic conversion of DL-MATI by *Pp*EST.

3.7. Optimum reaction pH and temperature

The highest activity of *Pp*EST were seen at pH 8–10 and 57–67 °C (Figs. 7 and 8) which is similar to that of esterase from *P. putida* MR-2068 at pH around 8 and near 70 °C [11].

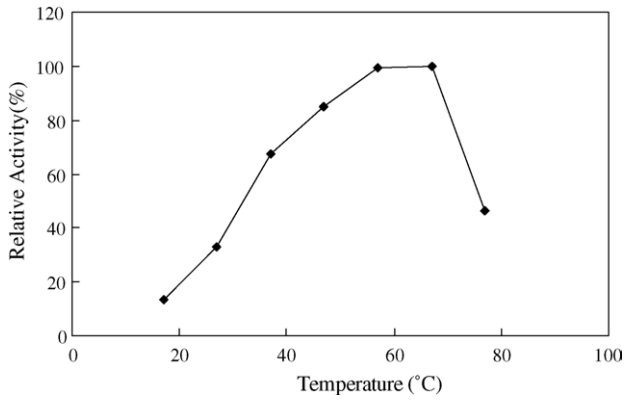


Fig. 8. Effect of temperature on enzymatic conversion of DL-MATI by *Pp*EST.

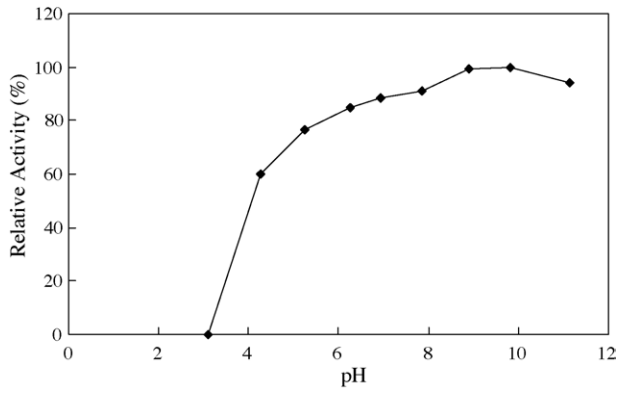


Fig. 9. pH stability of *Pp*EST.

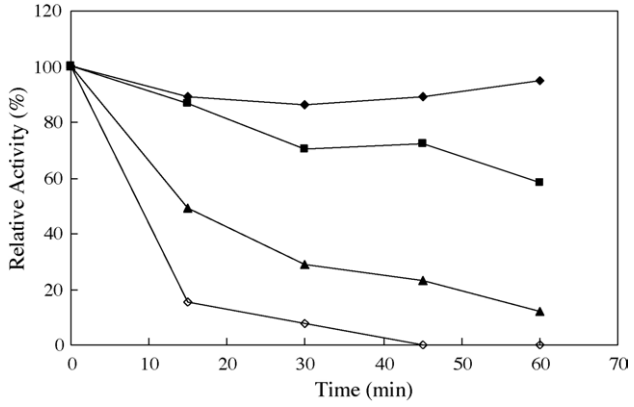


Fig. 10. Thermal stability of *Pp*EST at various temperatures: 57 °C (♦); 67 °C (■-); 72 °C (▲); 77 °C (◊).

3.8. pH stability and thermal stability

As shown in Fig. 9, *Pp*EST was fairly stable at alkaline pH but unstable at acidic pH and lost its activity completely at pH 3. As to the thermal stability of the enzyme (Fig. 10), it is expected that the stability was greater in lower than higher temperature. *Pp*EST remained stable for 1 h at 57 °C but lost its activity completely in 45 min at 77 °C.

4. Conclusion

To prepare an optically pure DAT from DL-MATI by enzymatic resolution method, it is necessary to select an enzyme that asymmetrically hydrolyzes the ester group and recognize the configuration at two-position. It was reported that the esterase from *P. putida* MR-2068 and the 3,4-dihydrocoumarin hydrolase from *A. calcoaceticus* F46 were able to hydrolyze methyl ester group of DL-MATI to give DAT with high optical purity [7,11]. In an effort to screen microorganisms that were capable of producing optical pure DAT from DL-MATI, several microorganisms were also identified which included *Pseudomonas fluorescens* (IFO12180, IFO3081 and IFO12055) and *P. putida* (IFO12996) [6] but none of the esterase gene from these strains were cloned, expressed and characterized. In this report, the *Pp*EST gene from *P. putida* IFO12996 was cloned by PCR method, its nucleotide sequence was revealed and it was expressed in *E. coli*. The deduced amino acid sequence exhibited high similarity to the esterase from *P. putida* MR-2068 [8] and 3,4-dihydrocoumarin hydrolase from *A. calcoaceticus* F46 [10]. The recombinant *Pp*EST from *E. coli* existed as a subunit molecular mass of 33 kDa. The kinetic constants (K_m and V_{max}) of *Pp*EST for DL-MATI were 34 mM and 1429 μmol/min/mg which were very similar to those of 3,4-dihydrocoumarin hydrolase from *A. calcoaceticus* F46 for DL-MATI [7] (26 mM and 1440 μmol/min/mg). *Pp*EST exhibited thermal and alkaline stability, which is similar to the esterase from *P. putida* MR-2068 [11].

DL-MATI has two functional groups in a molecule, i.e. a methyl ester and a thiol ester group. *Pp*EST like the esterase from *P. putida* MR-2068 and 3,4-dihydrocoumarin hydrolase from *A. calcoaceticus* F46 showed stereoselective hydrolysis of the methyl ester bond of DL-MATI to give DAT with high optical purity (97% e.e.). However, it has also been reported that the methyl ester bond of DL-MATI was unstable at pH above 7 and at temperature above 50 °C and this could cause the decrease of the optical purity of DAT prepared by the enzymatic resolution method [11]. In addition, the low solubility of DL-MATI in aqueous solution (40 mM) results in low product concentration, which limits its industrial application. To overcome this problem, a new substrate DL-ATIA with an amide functional group to replace the methyl ester group of DL-MATI was synthesized. The amide bond is more stable than the methyl ester bond under alkaline pH and high temperature, which can reduce the side reaction. Furthermore, the amide functional group also increases the aqueous solubility of DL-ATIA to 780 mM (compared to 40 mM of DL-MATI). DL-ATIA was asymmetrically hydrolyzed by *Pp*EST in quantitative manner and produced DAT in high optical purity (e.e. 97%). Enzyme kinetic analysis of DL-ATIA for *Pp*EST revealed that it was a poorer substrate than DL-MATI. However, it could be asymmetrically hydrolyzed by *Pp*EST in a reasonable time to yield a final product concentration 18-fold higher than that of DL-MATI. The higher final product concentration is one of the requirements for successful development of an industrial process. The enzymes and substrates that have been used for production of DAT or its analogs are summarized in Table 2. Three enzymes catalyzed resolution of DL-MATI with high stereoselectivity (e.e. value >97%) and DL-MATI was the most frequently used substrate for the enzymatic reaction. However, low water solubility of DL-MATI has limited its industrial application. To solve this problem, it can be achieved either by addition of emulsifier in two-phase system [13–14] or by engineering the substrate to make it more soluble in water [15]. In this report, substrate-engineering approach was chosen to prepare DL-ATIA to improve its water solubility.

*Pp*EST catalyzed the hydrolysis of both ester and amide bonds but it hydrolyzed the ester bond 255-fold more efficient than it hydrolyzed the amide bond. In other words, it has strong esterase activity and weak amidase activity. It has been reported that some amidase showed this characteristic [16]. The crystal structure of *Pp*EST recently was solved by us and it clearly shows the active site contain catalytic triad Ser97-Asp227-His256 but not the catalytic triad of amidase (Lys-Ser-Ser) [17].

Table 2
Enzymes and substrates used for production of DAT or its analogs

Enzyme	Substrate	Product	Yield (%)	e.e. (%)	Ref.
Mucor meihei lipase	Substrate 1	Product 1	35	80	4
Porcine pancreatic lipase	DL-MATI	Product 2	51	88	5
3,4-Dihydrocoumarin hydrolase (F46)	DL-MATI	DAT	49	99.9	7
Pseudomonas putida esterase (MR-2068)	DL-MATI	DAT	49.7	98.2	11
*Pp*EST (*Pseudomonas putida* IFO12996)	DL-MATI	DAT	45.4	97	
*Pp*EST (*Pseudomonas putida* IFO12996)	DL-ATIA	DAT	42.4	97	

Substrate 1: (±)-methyl-3-3′,5′-dimethoxybenzoylthio-2-methylpropionate; Product 1: (−)-3-3′,5′-dimethoxybenzoylthio-2*S*-methylpropionic acid; Product 2: (*R*)-(+)-methyl 3-mercapto-2-methylpropionate.

In addition, *Pp*EST does not contain the consensus sequence of the amidase signature family [16].

In conclusion, the *Pp*EST gene from *P. putida* IFO12996 was cloned and expressed in *E. coli*. *Pp*EST was able to asymmetric hydrolyze DL-MATI and DL-ATIA to give DAT with high optical purity. The higher stability and solubility of DL-ATIA in aqueous solution makes it a better choice for industrial production of DAT by enzymatic resolution method.

5. Accession numbers

The nucleotide sequence of esterase (*Pp*EST) from *P. putida* IFO12996 has been deposited in the GenBank under accession number DQ187948.

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