Journal of Molecular Catalysis B: Enzymatic 60 (2009) 138–144

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Enantioselective ester hydrolase from *Sphingobacterium* sp. 238C5 useful for chiral resolution of β-phenylalanine and for its β-peptide synthesis

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ARTICLE INFO

Article history:
Received 25 December 2008
Received in revised form 22 April 2009
Accepted 22 April 2009
Available online 3 May 2009

Keywords:
β-Amino acid
β-Amino acid ester hydrolase
(*S*)-β-Phenylalanine ethyl ester hydrolase
Sphingobacterium sp.
Chiral resolution
Transpeptidase
β-Peptide

ABSTRACT

A novel enzyme, β-phenylalanine ester hydrolase, useful for chiral resolution of β-phenylalanine and for its β-peptide synthesis was characterized. The enzyme purified from the cell free-extract of *Sphingobacterium* sp. 238C5 well hydrolyzed β-phenylalanine esters (*S*)-stereospecifically. Besides β-phenylalanine esters, the enzyme catalyzed the hydrolysis of several α-amino acid esters with L-stereospecificity, while the deduced 369 amino acid sequence of the enzyme exhibited homology to alkaline D-stereospecific peptide hydrolases from *Bacillus* strains. *Escherichia coli* transformant expressing the β-phenylalanine ester hydrolase gene exhibited an about 8-fold increase in specific (*S*)-β-phenylalanine ethyl ester hydrolysis as compared with that of *Sphingobacterium* sp. 238C5. The *E. coli* transformant showed (*S*)-enantiomer specific esterase activity in the reaction with a low concentration (30 mM) of β-phenylalanine ethyl ester, while it showed both esterase and transpeptidase activity in the reaction with a high concentration (170 mM) of β-phenylalanine ethyl ester and produced β-phenylalanyl-β-phenylalanine ethyl ester. This transpeptidase activity was useful for β-phenylalanine β-peptide synthesis.

1. Introduction

β-Phenylalanine (3-amino-3-phenylpropionic acid) is one of the β-amino acids present in several bioactive molecules, and β-phenylalanine and its derivatives are expected to form chiral building blocks for the synthesis of pharmaceutical agents [1,2]. For this reason, the stereoselective synthesis of β-phenylalanine and its derivatives have attracted the attention of many organic chemists and biochemists and several investigations have been performed [3–7]. Concerning the enzymatic kinetic resolution of β-phenylalanine, α-chymotrypsin [3], penicillin G acylase [4], lipase [5], and aminoacylase [8] have been reported to catalyze this reaction. There is recently interest in the use of β-aminotransferases [9]. However, the enantioselectivity and production yields were not satisfactory for practical purposes. In the previous study, microorganisms were discovered that can catalyze stereoselective β-phenylalanine ethyl ester (3-amino-3-phenylpropionic acid ethyl ester, BPAE) hydrolysis, and these microorganisms were utilized for the efficient synthesis of optically active BPAE from racemic BPAE [7]. *Sphingobacterium* sp. 238C5 was selected as the most potent strain with hydrolytic activity specific to (*S*)-BPAE and the enzyme catalyzing the hydrolysis was purified and characterized partially. The enzyme was distinguished from lipase Amano PS, which was the only previously reported enzyme catalyzing (*S*)-BPAE hydrolysis [5], because the enzyme did not catalyze the hydrolysis of several aliphatic carboxylate esters and aromatic carboxylate esters. In this article, the characteristics of this novel enzyme, (*S*)-stereospecific β-phenylalanine ester hydrolase [(*S*)-BEH], were investigated in detail. The present report describes the enzymological characterization and gene cloning of (*S*)-BEH from *Sphingobacterium* sp. 238C5 and heterologous expression of (*S*)-BEH in *Escherichia coli* resulting in the elucidation of the transpeptidase activity of the enzyme useful for β-phenylalanine β-peptide synthesis.

2. Materials and methods

2.1. Chemicals

Racemic BPAE, racemic 6-phenyl-2,4(1*H*,3*H*,5*H*)-pyrimidinedione, racemic 3-ureido-3-phenylpropionate, and racemic

Abbreviations: ADP, alkaline D-peptidase; BPAE, β-phenylalanine ethyl ester; DAC, D-alanyl-D-alanine carboxypeptidase; DEP, D-stereospecific endopeptidase; *e.e.*, enantiomer excess; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; LC–MS, liquid chromatography–mass spectrometry; PCR, polymerase chain reaction; (*S*)-BEH, (*S*)-β-phenylalanine ethyl ester hydrolase; *sbeh*, gene encoding (*S*)-β-phenylalanine ethyl ester hydrolase; UV, ultraviolet.

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doi:10.1016/j.molcatb.2009.04.011

4-phenylazetidine-2-one were kind gifts from Kaneka Co. Ltd. (Osaka, Japan). Racemic β-phenylalanine was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals used in this work were of analytical grade and commercially available.

2.2. Microorganisms and cultivation

Sphingobacterium sp. 238C5 was isolated as a BPAE-assimilating microorganism from a soil sample as reported in the previous study [7]. *Sphingobacterium* sp. 238C5 (=AKU 170), one of the strains preserved in the culture collection of the Faculty of Agriculture of Kyoto University, was cultivated for 24 h at 28 °C with shaking (300 strokes/min) in a medium comprising 1% tryptone, 1% yeast extract, 0.2% glucose, and 0.2% K_2HPO_4 (pH 7.0). For the expression of (*S*)-BEH in *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany), the transformant was cultivated for 1–2 h at 37 °C in Luria-Bertani medium and then 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the medium. The culture medium was further incubated for 20 h at 20 °C with shaking (300 strokes/min).

2.3. Enzyme assays

The standard methods for the activity assay were as follows. The reaction mixture contained, in 200 μl, 1.0% (w/v) BPAE, 200 mM potassium phosphate buffer (pH 6.0) and an appropriate amount of enzyme. The averages of three separate experiments, which were reproducible within 10%, are presented in the text and tables. Preparation of the purified enzyme was performed as described previously [7]. The mixture was incubated at 28 °C for 15–120 min and the reaction was terminated by adding 20 μl of 15% (v/v) perchloric acid. Investigations of the effects of pH, temperature, inhibitors, and metal ions on the enzymatic activity were carried out essentially by the standard methods with the slight modifications described below. The reaction mixture was centrifuged at 15,000 rpm for 3 min (MX-150, Tomy Seiko, Tokyo, Japan), and the supernatant was analyzed.

To evaluate the hydrolytic activity to racemic BPAE, the concentrations of BPAE and β-phenylalanine in the reaction mixture were analyzed by reverse-phase high-performance liquid chromatography (HPLC) by using a Shimadzu LC-VP system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil $5C_8$-MS column (0.46 mm × 250 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was acetonitrile–water (1:9 v/v, pH 2.0 adjusted with phosphoric acid) at a flow rate of 1.0 ml/min and the eluents were monitored at 210 nm by ultraviolet (UV) detection. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol β-phenylalanine per min from racemic BPAE under the above conditions. The analysis for enantiomeric purity of BPAE and β-phenylalanine was performed after derivatization with 2,3,4,6-tetra-*o*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [10–12]. The derivatized samples were analyzed by reverse-phase HPLC by using a Shimadzu LC-VP system equipped with Cosmosil $5C_{18}$-AR-II column (0.46 mm × 250 mm, Nacalai Tesque). The mobile phase was methanol–water (55:45 v/v, pH 2.5 adjusted with phosphoric acid) at a flow rate of 1.0 ml/min and the eluents were monitored at 250 nm by UV detection.

To evaluate the hydrolytic activity to α-amino acid esters and amides, the production of amino acids was analyzed by ion-exchange HPLC by using a Shimadzu LC-VP system equipped with TSK-gel QAE-2SW column (4.6 mm × 250 mm, Tosoh, Tokyo, Japan). The mobile phase was 6.6 mM potassium phosphate buffer pH 6.0 at a flow rate of 0.4 ml/min and the eluents were monitored at 210 nm by UV detection.

2.4. Preparation of internal peptides from the purified enzyme

Preparation of the purified enzyme was performed as described previously [7]. The enzyme solution containing 1 mg of the purified enzyme was lyophilized and dissolved in 100 μl buffer comprising 20 mM Tris/HCl (pH 9.0) and 8 M urea. After incubation at 37 °C for 60 min for denaturation by urea, the concentration of urea was decreased to 4 M by adding 100 μl of 20 mM Tris/HCl buffer (pH 9.0). After addition of 0.05 nmol of lysyl endopeptidase (Wako Pure Chemicals, Osaka, Japan), the solution was incubated at 30 °C for 6 h for digestion. The digested sample was applied to a μRPC C2/18 SC2/10 column (Amersham Pharmacia Biotech Co., Uppsala, Sweden) connected to a SMART system (Amersham Pharmacia Biotech Co.) and equilibrated with mobile phase of 0.1% (v/v) trifluoroacetic acid in water. The peptides were eluted with a linear gradient of 0–80% (v/v) acetonitrile in 10 ml of mobile phase at a flow rate of 0.1 ml/min. The amino acid sequences of the peptides were analyzed by automated Edman degradation with a 491HT protein sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Cloning of (S)-BEH gene, sbeh

The isolation of total DNA from *Sphingobacterium* sp. 238C5 was carried out by lysis of the cells with lysozyme (Wako Pure Chemicals) and proteinase K (Wako Pure Chemicals), extraction with phenol/chloroform (1:1 v/v), ethanol-precipitation, treatment with RNase, and re-precipitation with ethanol. Oligonucleotide primer pools were designed based upon the amino acid sequences of internal peptides: EDQQPVLIA and PLAHQYA. The primer pools used were a sense primer, GAIGAICA(A/G)CC(A/T/G/C)-GT(A/T/G/C)(C/T)T(A/T/G/C)AT(A/T/C)GC and an anti sense primer, GC(A/G)TA(C/T)TG(A/G)TG(A/T/G/C)GC(A/T/G/C)A(A/G)(A/T/G/C)GG. The DNA fragment was amplified by polymerase chain reaction (PCR) using a thermal cycler, T Gradient (Biometra, Gottingen, Germany). The PCR mixture comprised, in a total volume of 50 μl, 5 ng of genomic DNA of *Sphingobacterium* sp. 238C5 as a template, 200 pmol of each primer pool, 200 nmol of dNTPs, and 0.5 units of Ex Taq polymerase (Takara-Bio, Otsu, Japan). One thermal cycle consisted of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min for a total for 30 cycles. The PCR product was cloned into a pT7Blue vector (Novagen). Sequencing analysis of the insert fragment was carried out as described below. A genomic DNA library of *Sphingobacterium* sp. 238C5 was constructed using *E. coli*. DNA fragments obtained by the digestion of total DNA of *Sphingobacterium* sp. 238C5 with *Dra*I (Takara-Bio) were ligated into pBluescript SK+ vectors (Stratagene, La Jolla, CA, USA) digested with *Eco*RV (Takara-Bio). Colonies harboring a part of the *sbeh* gene were selected by colony hybridization using an AlkPhos Direct Labeling kit (Amersham Pharmacia Biotech Co.) and the PCR product described above as a probe, and the sequence of the insert fragment was analyzed. Further analysis of the full *sbeh* gene sequence was carried out by inverse PCR [13]. DNA fragments obtained from the digestion of genomic DNA of *Sphingobacterium* sp. 238C5 with *Xba*I (Takara-Bio) were circularized by self-ligation and used as a template. The oligonucleotide primers for inverse PCR were a sense primer, ATATCGGGAGCCCACAGCCTGTCGGGG, and an antisense primer, GAATTCCCGAAGTATGTGACAGCAAGTTC. The PCR conditions were the same as described above except that LA Taq polymerase (Takara-Bio) was used. The amplified DNA fragments were cloned into a pT7Blue vector and subjected to nucleotide sequencing. The nucleotides of the selected clones were sequenced by the dideoxy chain termination method, using a CEQ dye terminator cycle sequencing kit (Beckman Coulter) with an automated sequencer DNA Analysis System, CEQ2000XL (Beckman Coulter). Sequence data were analyzed with Genetyx-Mac 11.2 (Software Development, Tokyo, Japan).

2.6. Construction of E. coli expressing sbeh gene

The full-length *sbeh* gene was amplified by PCR using a T Gradient thermal cycler, with the following primers: 5′-ATGACTAGTCAGTTTCTGGATCTGAGATAC-3′ (the *Spe*I site is underlined) as the sense primer (nucleotide residues −40 to −19) and 5′-GCAGAGCTCCCAACTATTATTTTATTGCTG-3′ (the *Sac*I site is underlined) as the antisense primer (complementary to residues 1097–1126). The PCR conditions were the same as described above except that 20 cycles were performed. The PCR product was cloned into a pT7Blue vector and the insert fragment was confirmed to be accurate by sequencing analysis. The resultant plasmid was digested with *Nde*I (Takara-Bio) and *Sac*I (Takara-Bio), and the *sbeh* gene fragment was ligated into a pET-21a vector digested with *Nde*I and *Sac*I (pETSBEH). pETSBEH was introduced to *E. coli* BL21 (DE3) and the transformant was used in the subsequent experiment as the source of recombinant (*S*)-BEH. As a control, a pET-21a vector without an insert fragment was used to transform *E. coli* BL21 (DE3).

2.7. Analysis of recombinant (S)-BEH activity

Cells of *E. coli* BL21 (DE3) harboring pETSBEH or pET-21a and cells of *Sphingobacterium* sp. 238C5 were obtained from the cultures described above, washed with physiological saline, and used as washed cells. The enzyme activity was analyzed in resting cell reactions containing 30 mM racemic BPAE as the substrate and 10% (w/v) wet cells as the catalysts in 200 mM potassium phosphate buffer (pH 6.0). After incubation at 28 °C, aliquots of the reaction mixtures were withdrawn and centrifuged at 15,000 rpm for 3 min (MX-150), and then the supernatant were analyzed by reverse-phase HPLC as described above.

2.8. Liquid chromatography–mass spectrometry (LC–MS) analysis

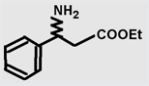
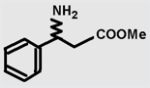
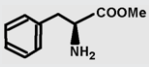
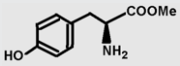
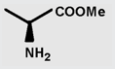
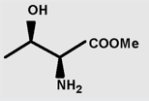
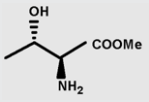
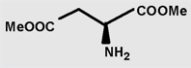
The HPLC system was equipped with a photodiode array detector, SPD-M10A (Shimadzu) and a mass detector, SHIMADZU LCMS 2010A (Shimadzu) in series with a HPLC binary pump, autosampler, and degasser controlled by LCMS solution software (Shimadzu). The mass detector was equipped with an atmospheric pressure chemical ionization system (capillary voltage, 4.5 kV; capillary temperature, 400 °C; dry gas (N_2) temperature, 200 °C). Mass scans were measured in the range of *m*/*z* 50–500 in positive ionization mode. Chromatographic separation was carried out on reverse-phase HPLC by using Cosmosil $5C_{18}$-AR-II column. The mobile phase was acetonitrile–water (3:7 v/v, 0.1% trifluoroacetic acid) at a flow rate of 0.5 ml/min and the eluents were monitored by UV detection at 210 nm. The HPLC and mass spectrometry data were integrated and analyzed by the LCMS solution software.

3. Results and discussion

3.1. Substrate specificity of (S)-BEH

To investigate the substrate specificity, the purified (*S*)-BEH enzyme was used to hydrolyze 20 mM initial concentrations of the following compounds: BPAE, β-phenylalanine methyl ester, α-amino acid esters, α-amino acid amides, and amide derivatives of β-phenylalanine and the activity was assayed as described in Section 2. The substrates, β-phenylalanine methyl ester, L-phenylalanine methyl ester, L-tyrosine methyl ester, L-alanine methyl ester, L-threonine methyl ester, L-*allo*-threonine methyl ester, and L-aspartic acid dimethyl ester were hydrolyzed by the purified enzyme with relative activities of 105%, 25.6%, 79.2%, 273%, 59.8%, 200%, and 59%, respectively, to the activity with racemic BPAE set as 100% (0.26 units) (Table 1). Extremely low levels of activity or spontaneous chemical hydrolysis were observed with D-α-amino acid esters, indicating that they were not hydrolyzed by the enzyme and the enzyme is L-specific in α-amino acid esters hydrolysis. α-Amino acid amides, and amide derivatives of β-phenylalanine were not hydrolyzed by (*S*)-BEH.

Table 1
Substrate specificity of (*S*)-BEH. The reactions were carried out under the conditions as described in Section 2 except that the indicated compounds were substituted for racemic BPAE. 0.26 units of the purified (*S*)-BEH was used for each reaction. The following compounds were not hydrolyzed or extremely less hydrolyzed: D-phenylalanine methyl ester, D-tyrosine methyl ester, D-alanine methyl ester, D-threonine methyl ester, glycine ethyl ester, L-phenylalanine amide, D-phenylalanine amide, L-tyrosine amide, D-tyrosine amide, racemic 6-phenyl-2,4(1*H*,3*H*,5*H*)-pyrimidinedione, racemic 3-ureido-3-phenylpropionate, and racemic 4-phenylazetidine-2-one.

Substrate	Chemical structure	Relative activity (%)
Racemic BPAE		100
Racemic β-phenylalanine methyl ester		105
L-Phenylalanine methyl ester		25.6
L-Tyrosine methyl ester		79.2
L-Alanine methyl ester		273
L-Threonine methyl ester		59.8
L-*allo*-Threonine methyl ester		200
L-Aspartic acid dimethyl ester		59.0

3.2. Stereoselectivity of (S)-BEH

The stereoselectivity of (*S*)-BEH in racemic BPAE hydrolysis was analyzed by the standard methods, except that the substrate concentration was 20 mM. In a 3 h reaction, half of the 20 mM racemic BPAE was hydrolyzed and the optical purity of the remaining BPAE was reached 99% *e.e.* for (*R*)-BPAE. This result indicated that the purified enzyme catalyzed (*S*)-BPAE specific hydrolysis.

From the viewpoint of stereoselectivity for the amino group, (*S*)-BEH showed a preference for (*S*)-isomers, such as (*S*)-β-phenylalanine esters and L-α-amino acid esters (=(*S*)-α-amino acid esters). L-Threonine ester and L-*allo*-threonine ester were good substrates of (*S*)-BEH but D-threonine ester was not. These results suggested that (*S*)-BEH recognized the configuration of the amino group irrespective of its position, while recognition of that of hydroxyl group in β-position was less strict.

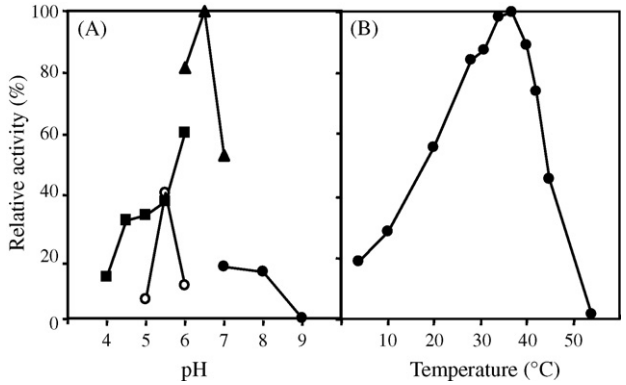


Fig. 1. Effects of pH and temperature on the activity of (*S*)-BEH. Effects of pH (A) and temperature (B) on the enzymatic activity are shown. (A) Effects of pH were assayed by the method given in the text. The pH of the reaction mixture was adjusted by addition of the following buffers: acetate/sodium acetate buffer (■) for pH 4.0–6.0, MES/NaOH buffer (○) for pH 5.0–6.0, potassium phosphate buffer (▲) for pH 6.0–7.0, Tris/HCl buffer (●) for pH 7.0–9.0. The reaction mixture was incubated at 28 °C for 30 min. (B) The effects of temperature are analyzed by the method given in the text. The reaction mixture was incubated at pH 6.0 for 30 min.

3.3. Effects of pH and temperature on the enzyme

The optimal pH for the activity was investigated. The enzyme showed maximal activity at pH 6.5 (Fig. 1A). The enzyme reactions were examined at various temperatures (4–54 °C) at pH 6.0 and the enzyme activity was found to be maximal at 37 °C (Fig. 1B).

The stability of the enzyme was examined at various pH values. The enzyme was incubated at 28 °C for 30 min under various pHs, and then the residual activity of the enzyme was assayed by standard methods. The enzyme was stable in the pH range 6.0–9.0 (Fig. 2A). The stability of the enzyme was examined at various temperatures (0–90 °C) for 30 min at pH 6.0. The enzyme was stable below 37 °C and 10% of the original activity was retained at 45 °C (Fig. 2B).

3.4. Effects of inhibitors and metal ions on the enzymatic activity

The effects of various inhibitors and metal ions (2 mM) on the enzyme activity were examined. As a result of analysis by the standard methods, the enzyme activity was inhibited by Ag^{+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and 5,5′-dithiobis-2-nitrobenzoate by 0.64%, 2.4%, 22%, 39%, 39%, and 46% of the original activity, respectively. Metal ion chelaters (ethylenediaminetetraacetic acid, 8-hydroxyquinoline, and *o*-phenanthroline), serine protease inhibitors (phenylmethane sulfonyfluoride and diisopropylphosphofluoride), carbonyl reagents (hydroxylamine, phenylhydrazine, and semicarbazide), and sulfhydryl inhibitors except for 5,5′-dithiobis-2-nitrobenzoate (iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide) showed no significant influence on the activity.

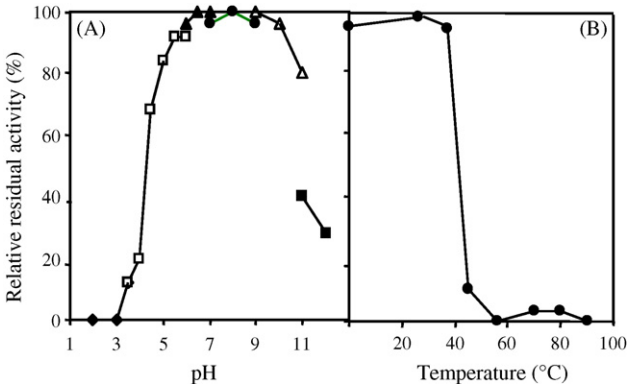


Fig. 2. Effects of pH and temperature on the stability of (*S*)-BEH. The effects of pH (A) and temperature (B) on the stability are shown. (A) The effects of pH were assayed by the method given in the text. The purified enzyme was incubated at 28 °C for 30 min after pH was adjusted by addition of the following buffers; sodium acetate/HCl buffer (♦) for pH 2.0–3.5, acetate/sodium acetate buffer (□) for pH 3.5–6.0, potassium phosphate buffer (▲) for pH 6.0–7.0, Tris/HCl buffer (●) for pH 7.0–9.0. Borate/NaOH buffer (△) for pH 9.0–11.0, Na_2PO_4/NaOH buffer (■) for pH 11.0–12.0. After this pH treatment, the residual activity was assayed. (B) Effects of temperature were analyzed by the method given in the text. The purified enzyme was incubated at pH 6.0 for 30 min under various temperature conditions and the residual activity was assayed.

3.5. Analysis of amino acid sequences

The *N*-terminal amino acid sequence of the enzyme could not be determined. Ten internal peptides were obtained and their sequences were determined as follows: W-K, R-S-I-D-I-G-S-P-Q-P-V-G-E-K, G-F-Q-H-V-I-N-P-F-I-G-K, D-A-I-G-I-I-V-H-V-E-A-P-K, E-T-S-L-F-F-Q-Y-L-F-N-G-K, F-S-Y-A-D-I-N-Y-L-L-L-T-E-I-I-E-Q-K, Y-N-W-D-S-Y-A-I-D-P-S-W-D-L-Y-G-G-G-G-G-I-A-A-T-A-K, T-W-F-I-N-L-E-H-Y-P-E-S-T-L-P-L-A-H-Q-Y-A-N-K, N-L-L-S-H-T-S-G-I-Q-D-Y-V-D-E-A-Y-F-E-F-V-N-Q-H-P-K, and H-N-I-S-W-S-Y-A-R-G-V-A-D-S-T-N-Q-A-L-E-D-Q-Q-P-V-L-I-A-S-N-T-K.

3.6. Cloning and sequence analysis of the gene encoding (*S*)-BEH (*sbeh*)

The obtained sequences were integrated and the full length of the ORF sequence encoding the *sbeh* gene was determined (Fig. 3). The ORF of the *sbeh* gene consisted of 1110 bp with an initiation codon (ATG) and a stop codon (TAA). A probable ribosome-binding sequence, AGAAAAG, was present 5 bases upstream of the putative translational start codon. The ORF encodes a protein consisting of 369 amino acid residues. All the amino acid sequences determined for peptide fragments of *Sphingobacterium* sp. 238C5 (*S*)-BEH exactly matched those predicted from the DNA sequence. The ORF encodes a protein consisting of 369 amino acid residues. The predicted molecular mass was 42,270 Da, which is in good agreement with the molecular mass of the purified enzyme determined by gel-filtration chromatography (42,000–45,000) [7].

3.7. Comparison of the amino acid sequence of the enzyme

As the result of homology searches in databases of known proteins using BLAST, the deduced amino acid sequence of (*S*)-BEH showed similarity to penicillin-binding proteins or β-lactamases. In particular, the highest similarity was observed in these enzymes from *Bacillus* strains. The deduced amino acid sequence of (*S*)-BEH showed about 25% identity over 290 amino acid residues with the alkaline D-peptidase from *Bacillus anthracis*, the D-specific endopeptidase from *Bacillus cereus*, and D-alanyl-D-alanine carboxypeptidase from *Bacillus thuringiensis* (Fig. 4) [14–16].

The reaction mechanism and the active site of penicillin-binding proteins, β-lactamases [17–20], and D-specific endopeptidases [21,22] have been investigated extensively. The catalytic center of these enzymes is defined by three amino acid motifs. Motif 1, S-X-X-K (where S is the essential serine residue and X is a variable amino acid residue) occupies a central position in the catalytic center. Motif 2, [S/Y]-X-[N/C], defines one side of the active site, and motif 3, [K, H]-[T, S]-G, defines the other side [20]. In an alignment of (*S*)-BEH with the sequences of alkaline D-peptidase from *Bacillus anthracis*, D-specific endopeptidase from *Bacillus cereus*, and D-alanyl-D-alanine carboxypeptidase from *Bacillus thuringiensis*, motif 1 was highly conserved in (*S*)-BEH between residue 78–81 (Fig. 4). However, motif 2 and motif 3 were not conserved. These findings led to the speculation that (*S*)-BEH is related to penicillin-

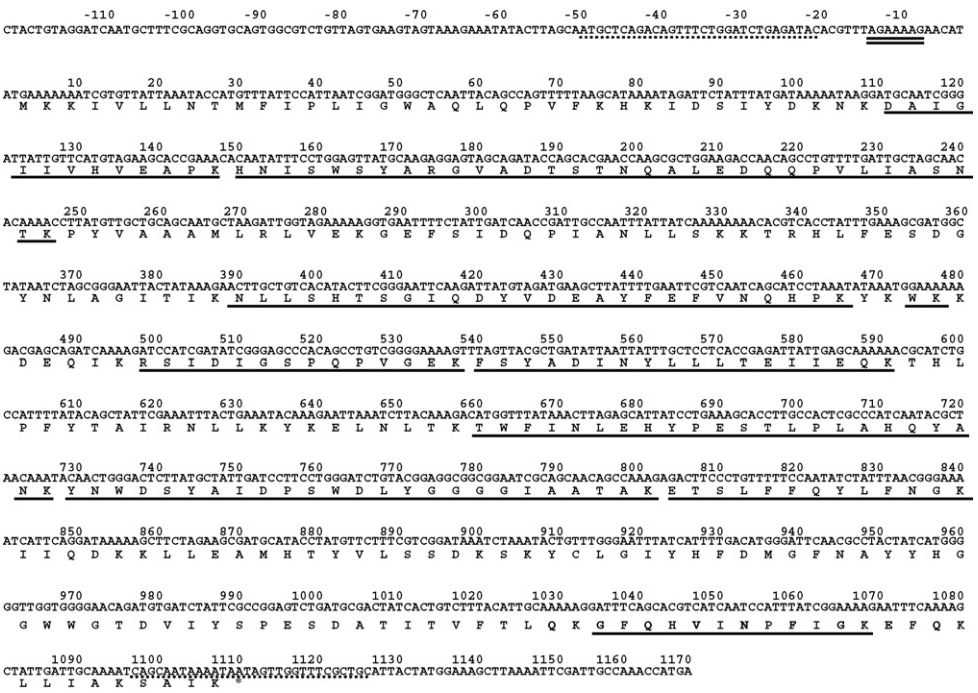


Fig. 3. Nucleotide and deduced amino acid sequences of the *sbeh* gene and flanking regions. A putative Shine-Dalgarno sequence is *double underlined*. The *asterisk* denotes a translational stop codon. Amino acid sequences that are identical to those of the internal peptides prepared from purified enzyme are *underlined*. Primer sequences used for construction of expression vector are *dotted underline*. The novel nucleotide sequence data published here have been submitted to the DDBJ and are available under accession number AB465041.

sensitive D-alanine carboxypeptidases or penicillin-inactivating β-lactamases, but the reaction mechanism and the catalytic site of this enzyme are different. This was supported by the fact that (*S*)-BEH hydrolyzes α-amino acid esters L-stereospecifically, while penicillin-binding protein and β-lactamase have mainly D-stereospecificity.

3.8. Heterologous expression of the sbeh gene in E. coli BL21 (DE3)

The expression vector pETSBEH was constructed as described above and was introduced into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) harboring pETSBEH, *E. coli* BL21 (DE3) harboring pET-21a as control, and *Sphingobacterium* sp. 238C5 were cultivated and the (*S*)-BEH

Fig. 4. Comparison of the deduced amino acid sequence of (*S*)-BEH from *Sphingobacterium* sp. 238C5 with alkaline D-peptidase (ADP) from *Bacillus anthracis*, D-stereospecific endopeptidase (DEP) from *Bacillus cereus*, and D-alanyl-D-alanine carboxypeptidase (DAC) from *Bacillus thuringiensis*. Homology searches were carried out as described in the text. Gaps in the aligned sequences are indicated by *dashes*. *Asterisks* indicate highly conserved amino acid residues. The serine residue and the lysine residue contained in deduced motif 1 (S-X-X-K), which form the catalytic center, are closed in *boxes*.

activities of the washed cells prepared from these strains were evaluated. The cells of *E. coli* BL21 (DE3) harboring pETSBEH cultured with or without IPTG and cultured *Sphingobacterium* sp. 238C5 showed (*S*)-BEH activity at 0.88 mU/mg of cells, 0.25 mU/mg of cells, and 0.11 mU/mg of cells, respectively. The cells of *E. coli* BL21 (DE3) harboring pET-21a as control had no (*S*)-BEH activity.

3.9. Catalytic properties of the E. coli transformant

The recombinant enzyme showed stereoselectivity for the (*S*)-isomer in the resting cell reaction with 30 mM racemic BPAE as the substrate and chiral resolution was achieved stoichiometrically within 3 h. In 25 h reaction with 100 mM racemic BPAE, 42.5 mM of BPAE was hydrolyzed but produced BPA was 15.8 mM (Fig. 5A). In the reverse-phase HPLC analysis of the reaction mixtures, a by-product with higher hydrophobicity was detected (Fig. 5B).

The amount of β-phenylalanine produced was less than that expected from the amount of hydrolyzed BPAE, and the production of an unknown compound besides BPAE and β-phenylalanine was observed on HPLC chromatograms. LC–MS analysis of the reaction mixture was performed and the mass spectrum corresponding to the unknown peak showed a characteristic mass ion peak with the molecular mass of 341 (Fig. 6B), indicating that the product was ethyl ester of β-phenylalanine dipeptide, β-phenylalanyl-β-phenylalanine ethyl ester. β-Phenylalanyl-β-phenylalanine ethyl ester was the only one product other than BPA in the reaction with

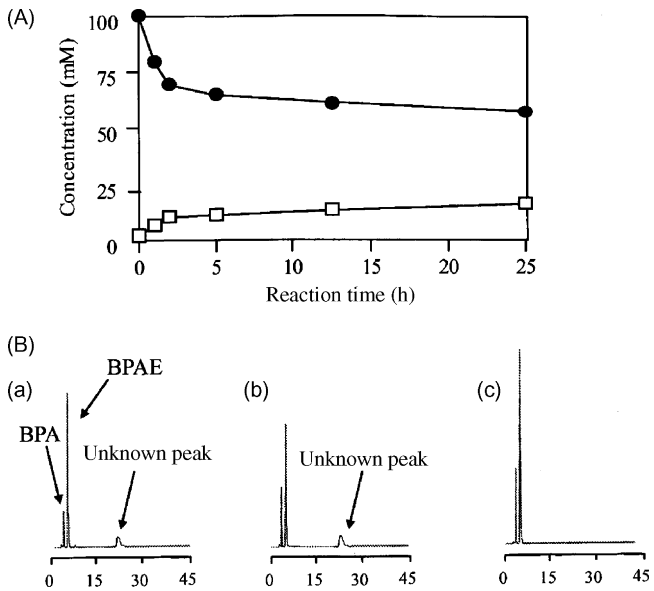


Fig. 5. Time course of the reaction with racemic BPAE and the reverse-phase HPLC chromatograms of the reaction mixtures. (A) Time course of the reaction with 100 mM racemic BPAE as the substrate was analyzed as described in the text. Concentration of BPA (●) and BPAE (□) are shown. (B) The reaction mixture was analyzed as described in the text. Chromatogram (a), the reaction mixture after 2-h incubation; Chromatogram (b), the reaction mixture after 25-h incubation; Chromatogram (c), the reaction mixture without the enzyme after 25-h incubation.

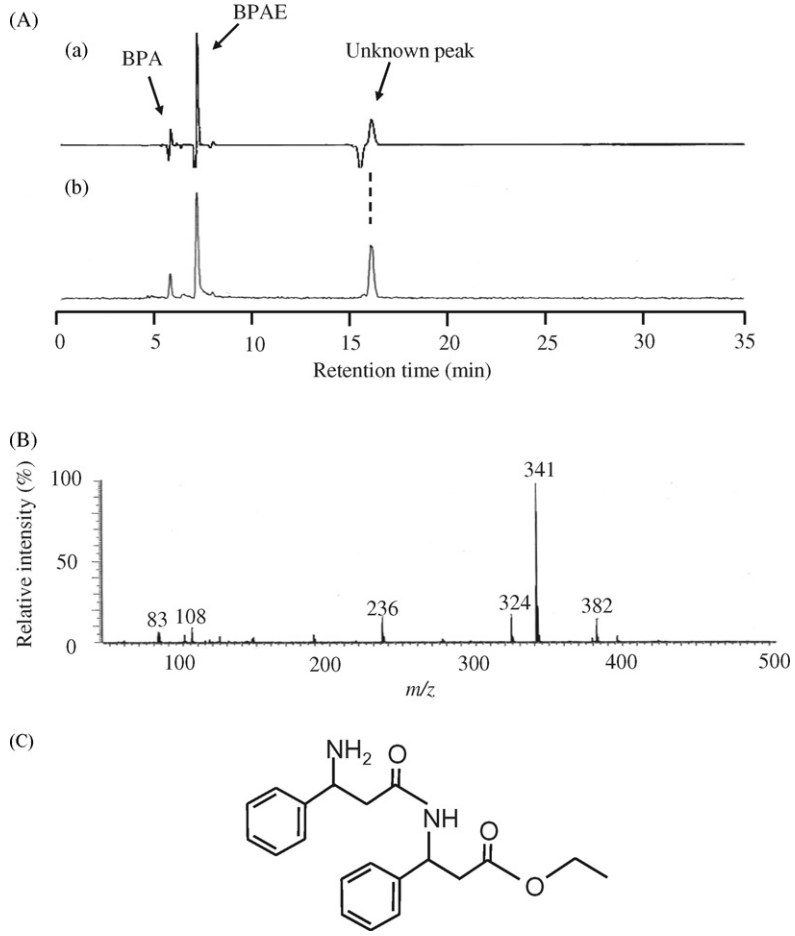


Fig. 6. LC–MS analysis of the unknown compound in the (*S*)-BEH-catalyzed reaction. The reaction mixture was analyzed by LC–MS as described in the text, and the chromatograms obtained are shown in A. (a) HPLC chromatogram detected by UV detection at 210 nm, (b) total ion mass chromatogram. Mass spectrum obtained from compound with a retention time of 15.5 min (corresponding to the unknown peak) is shown in B. The proposed structure of the unknown compound is shown in C. BPA: β-phenylalanine; BPAE: β-phenylalanine ethyl ester.

BPAE as the substrate, and the dipeptide ester accumulated in the course of the reaction and not decomposed.

A recent report by Heck et al. [23] of the β-peptidyl aminopeptidases, BapA from *Sphingosinicella xenopeptidilytica* 3-2W4, and DmpA from *Ochrobactrum anthropi* LMG7991 which were applied for the synthesis of β-peptides encouraged us to continue investigations into the functional analysis of the new activity of the (*S*)-BEH. Interestingly, the *E. coli* transformant showed esterase activity in the reaction with a low concentration (30 mM) of BPAE, while it showed both esterase and transpeptidase activity in the reaction with a high concentration (170 mM) of BPAE and produced β-phenylalanyl-β-phenylalanine ethyl ester (Fig. 6C). We previously reported the kinetic constants of the enzyme activity toward racemic BPAE and BPA methyl ester [7]. K_m values for racemic BPAE was 13 mM. This suggested that ratio of these activities is intensively influenced by the substrate concentration. Many penicillin-binding protein and β-lactamase, which belong to serine-acyl transferase family, have been reported to show esterase activity, peptidase activity, and transpeptidase activity. The reaction mechanisms of esterase activity and transpeptidase activity in acyl-serine transferase involving β-lactamases and penicillin-binding proteins are essentially identical. The electrophilic group, R_1-CO-X-R_2, is transferred to an acceptor, H-Y via the formation of a serine-ester-linked acyl enzyme intermediate. X donates an oxygen atom, a sulfur atom or NH group. When H-Y is H_2O, the carbonyl donor is hydrolyzed and the product is R_1-COOH. When H-Y is an amino compound, NH_2-R_3, the carbonyl donor is transpeptidated and the product is R_1-CO-NH-R_3 [20]. The transpeptidation occurred with high concentrations of amino compounds. In case of (*S*)-BEH, transpeptidation was detected with much higher concentrations of BPAE (more than 100 mM) than the K_m value of (*S*)-BEH for BPAE (13 mM). These findings supported the suggestion that (*S*)-BEH not only has esterase activity but also transpeptidase activity, and that the ethyl ester of β-phenylalanine dipeptide was produced by this transpeptidase activity.

4. Conclusions

Through this study, it was revealed that (*S*)-BEH is unique both in its function and primary structure as listed below:

(1) (*S*)-BEH well hydrolyzed β-phenylalanine esters (*S*)-stereospecifically. Besides β-phenylalanine esters, the enzyme catalyzed the hydrolysis of several α-amino acid esters with L-stereospecificity.
(2) The primary structure of (*S*)-BEH showed similarity to putative penicillin-binding proteins or penicillin-inactivating β-lactamases that show mainly D-stereospecific activity in α-amino acid esters hydrolysis, while (*S*)-BEH hydrolyzed several α-amino acid esters with L-stereospecificity and had different active site structure.
(3) (*S*)-BEH showed esterase activity in the reaction with a low concentration of BPAE, while it showed both esterase and transpeptidase activity in the reaction with a high concentration of BPAE and produced β-phenylalanyl-β-phenylalanine ethyl ester.

These results clearly showed that (*S*)-BEH is useful for chiral resolution of β-phenylalanine and for its β-peptide synthesis.

Acknowledgements

This work was supported partially by COE for Microbial-Process Development Pioneering Future Production Systems (to S.S.) from the Ministry of Education, Science, Sports, and Culture of Japan and by the Project for the Development of a Technological Infrastructure for Industrial Bioprocesses in R&D of New Industrial Science and Technology Frontiers (to S.S.) from the New Energy and Industrial Technology Development Organization of Japan.

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