

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 β -Aryl- β -amino acid aminotransferase from *Variovorax* sp. JH2 is useful for
2 enantioselective β -phenylalanine production

3
4 Makoto Hibi^a, Junichi Mano^b, Tairo Hagishita^a, Jun Shima^c, Sakayu Shimizu^b, and Jun
5 Ogawa^{b, *}

6
7 ^aIndustrial Microbiology, Graduate School of Agriculture, Kyoto University,
8 Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

9 ^bDivision of Applied Life Sciences, Graduate School of Agriculture, Kyoto University,
10 Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

11 ^cResearch Division of Microbial Sciences, Kyoto University, Kitashirakawa-oiwakecho,
12 Sakyo-ku, Kyoto 606-8502, Japan

13
14 *Corresponding author. Tel: +81-75-753-6122, Fax: +81-75-753-6128
15 E-mail address: ogawa@kais.kyoto-u.ac.jp (J. Ogawa)

16
17 Abbreviations: BHA, glutamate-1-semialdehyde 2,1-aminomutase from *Bacillus*
18 *halodurans*; CDI, glutamate-1-semialdehyde 2,1-aminomutase from *Corynebacterium*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 *diphtheriae*; CNE, glutamate-1-semialdehyde 2,1-aminomutase from *Cryptococcus*
- 2 *neoformans*; FPLC, fast protein liquid chromatography; FRA, aminotransferase class-III
- 3 from *Frankia* sp.; *e.e.*, enantiomer excess; GITC,
- 4 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl isothiocyanate; HPLC, high-performance
- 5 liquid chromatography; IP, internal peptide; PLP, pyridoxal 5'-phosphate; (*S*)-BAT,
- 6 (*S*)- β -phenylalanine:2-oxoglutarate aminotransferase; SDS-PAGE, sodium dodecyl
- 7 sulfate-polyacrylamide gel electrophoresis; SPO, glutamate-1-semialdehyde
- 8 2,1-aminomutase from *Schizosaccharomyces pombe*.

1
2 **1 ABSTRACT**
3
4

5 2 A bacterium, *Variovorax* sp. JH2, has an ability to degrade (*S*)- β -phenylalanine
6
7
8 3 stereoselectively. The enzyme involved in the degradation,
9
10
11 4 (*S*)- β -phenylalanine:2-oxoglutarate aminotransferase, was purified to homogeneity from
12
13
14 5 *Variovorax* sp. JH2 and characterized. The enzyme was useful for (*R*)- β -phenylalanine
15
16
17
18 6 production from racemic β -phenylalanine by enantioselective decomposition of
19
20
21 7 (*S*)- β -phenylalanine through transamination. (*S*)- β -Phenylalanine and
22
23
24 8 (*S*)-3-amino-3-(3-pyridyl)propionate served as good amino-donors in the transamination
25
26
27 9 and 2-oxoglutarate, oxaloacetate, pyruvate, and 1,3-acetonedicarboxylate served as
28
29
30 10 amino-acceptors. The enzyme had a molecular weight of about 72,000 and consisted
31
32
33
34 11 of two identical subunits. Three internal amino acid sequences (54, 67, and 63
35
36
37 12 residues) were determined and showed homology with glutamate-1-semialdehyde
38
39
40 13 2,1-aminomutases.
41
42
43
44 14

45
46 15 **Key words:** β -Phenylalanine aminotransferase; β -Aryl- β -amino acid aminotransferase;
47
48
49 16 *Variovorax* sp.; Stereoselective synthesis
50
51
52
53 17

1. Introduction

β -Phenylalanine (3-amino-3-phenylpropionic acid) is a type of β -amino acid present in several bioactive molecules, such as antibiotics and enzyme inhibitors [1, 2]. Because of its bioactivity, optically active β -phenylalanine and its derivatives are becoming important chiral building blocks for the synthesis of pharmaceutical agents. Enzymes catalyzing the synthesis reaction of β -phenylalanine are one of the potential methods for the preparation of chiral compounds, however, only a few enzymatic methods for the synthesis of optically active β -phenylalanine and its derivatives have been developed [3-7]. In our previous report, the microbial production of optically active β -phenylalanine through stereoselective degradation of racemic β -phenylalanine was reported [8]. Microorganisms were discovered that can catalyze stereoselective β -phenylalanine degradation available for the synthesis of optically active β -phenylalanine, and efficient β -phenylalanine production was achieved using these potent strains. In particular, a potent strain *Variovorax* sp. JH2 isolated as a β -phenylalanine-assimilating microorganism showed an ability to degrade (*S*)- β -phenylalanine stereoselectively through transamination with 2-oxoglutarate as an amino-acceptor. Some enzymes involved in β -amino acid metabolism have been reported, such as ω -amino acid aminotransferases and leucine 2,3-aminomutase [9-14].

1 However, there are a few reports on β -phenylalanine aminotransferases [6, 7]. The
2 present study investigated the aminotransferase involved in β -phenylalanine metabolism
3 of *Variovorax* sp. JH2. The potential of the purified enzyme for (*R*)- β -phenylalanine
4 production from racemic β -phenylalanine by enantioselective decomposition of
5 (*S*)- β -phenylalanine through transamination in the presence of 2-oxoglutarate was
6 examined and the detailed characterization of the enzyme,
7 (*S*)- β -phenylalanine:2-oxoglutarate aminotransferase ((*S*)-BAT), is described.

9 **2. Materials and methods**

10 *2.1. Chemicals*

11 DL-3-Amino-3-phenylpropionic acid (racemic β -phenylalanine) was purchased from
12 Aldrich Chemical Co. (Milwaukee, WI, USA). 3-Oxo-3-phenylpropionic acid
13 (benzoylactic acid) was a kind gift from Kaneka Co., Ltd (Osaka, Japan). All other
14 chemicals used in this work were of analytical grade and available commercially.

16 *2.2. Microorganisms and cultivation*

17 *Variovorax* sp. JH2 isolated from a soil sample as a β -phenylalanine-assimilating
18 microorganism was used as a source of enzyme. The strain was cultivated at 28°C

1
2 1 with shaking (120 strokes/min) for 24 h in 2-L flasks containing 500 ml of medium
3
4
5 2 comprising 0.1% (w/v) KH_2PO_4 , 0.1% (w/v) K_2HPO_4 , 0.03% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,
6
7
8 3 0.01% (w/v) yeast extract, 0.2% (w/v) NH_4Cl , and 0.3% (w/v) racemic β -phenylalanine
9
10
11 4 (pH 7.0).
12
13
14
15 5
16
17

18 2.3 *Enzyme assays*

20
21 7 The standard methods for the activity assay were as follows. The 200 μl reaction
22
23
24 8 mixture contained 10 mM racemic β -phenylalanine, 10 mM 2-oxoglutarate, 5 mM
25
26
27 9 pyridoxal-5'-phosphate (PLP), 200 mM Tris/HCl buffer (pH 9.0), and an appropriate
28
29
30 10 amount of enzyme. The mixture was incubated at 28°C for 15 to 120 min where the
31
32
33
34 11 reaction proceeded linearly, and the reaction was terminated by adding 20 μl of 15%
35
36
37 12 (v/v) perchloric acid. Investigations of the effects of pH, temperature, and chemicals
38
39
40 13 on the enzymatic activity and kinetic parameters were carried out essentially by the
41
42
43 14 standard methods with slight modifications as described above. The reaction mixture
44
45
46 15 was centrifuged at 15,000 rpm for 3 min (MX-150; Tomy Seiko, Tokyo, Japan), and the
47
48
49 16 supernatant was analyzed as described below.
50
51
52
53 17
54
55
56 18 2.4. *Analytical methods for enzyme activity*
57
58
59
60
61
62
63
64
65

1 To evaluate the enzymatic activity in the transamination of β -phenylalanine with
2 2-oxoglutarate, the concentration of β -phenylalanine and benzoylactic acid in the
3 reaction mixture was analyzed by reverse-phase HPLC by using a Shimadzu LC-VP
4 system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil 5C₁₈-AR-II column (0.46 ×
5 250 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase was
6 acetonitrile/water/trifluoroacetate (4/6/0.1, v/v/v) at a flow rate of 1.0 ml/min and the
7 effluents were monitored at 210 nm by ultraviolet detection. One unit (U) of enzyme
8 activity was defined as the amount catalyzing the formation of 1 μ mol benzoylactic
9 acid per min from racemic β -phenylalanine under the above conditions. To investigate
10 the substrate specificity for the amino-acceptor, the decrease in β -phenylalanine
11 concentration by transamination with β -phenylalanine as an amino-donor and various
12 amino-acceptors was monitored by the same method. To investigate the substrate
13 specificity for the amino-donor, the formation of L-glutamate by transamination with
14 2-oxoglutarate as an amino-acceptor and various amino-donors was monitored using an
15 amino acid analyzing system consisting of Separations module 2695 (Waters, Milford,
16 MA, USA) and Multi λ fluorescence detector 2475 (Waters). Samples were analyzed
17 after derivatization with *N*-hydroxysuccinimidyl-6-aminoquinolinylcarbamate. The
18 effluents were monitored by fluorescence detection (excitation, 250 nm; emission, 395

1 nm).

2 Analysis of the enantiomeric purity of the remaining β -phenylalanine was performed
3 using reverse-phase HPLC after derivatization with
4 2,3,4,6-tetra-*o*-acetyl- β -D-glucopyranosyl isothiocyanate (GITC) [15-17].
5 Methanol/water (55/45, v/v, pH 2.5 adjusted with phosphoric acid) was used as the
6 mobile phase. Investigation of stereospecificity towards
7 3-amino-3-(3-pyridyl)propionate was also performed by the same method.

8 9 *2.5. Analytical methods for proteins*

10 Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules,
11 CA, USA) with bovine serum albumin as a standard [18]. To assay the relative
12 concentration of protein eluted by liquid chromatography, the effluents were monitored
13 at 280 nm by ultraviolet detection.

14 The relative molecular mass of the native enzyme was determined by gel-filtration
15 liquid chromatography. This method was carried out using a fast protein liquid
16 chromatography (FPLC) system (Amersham Pharmacia Biotech Co., Uppsala, Sweden)
17 equipped with Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech Co.).
18 The mobile phase comprised 200 mM NaCl and 20 mM potassium phosphate buffer

1 (pH 7.0) and the flow rate was 0.3 ml/min. Partially purified enzyme solution was
2 injected and the eluate was fractionated at 0.3 ml/tube. The molecular mass of the
3 enzyme was calculated based on the relative mobility using standard proteins (M.W.
4 marker; Oriental Yeast Co., Tokyo, Japan).

5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was
6 performed in a 12.5% polyacrylamide gel using a Tris/glycine buffer system [19]. The
7 molecular mass of the subunit of the enzyme was determined from a comparison of the
8 mobility of the purified enzyme in SDS-PAGE with those of standard proteins (M.W.
9 marker III; Daiichi Kagaku Yakuhin, Tokyo, Japan).

10

11 2.6. Enzyme purification

12 All procedures were carried out at 0-5°C, and 20 mM potassium phosphate buffer
13 (pH 7.0) containing 0.2 mM PLP was used as a standard buffer unless otherwise
14 specified. *Variovorax* sp. JH2 cells (about 28 g obtained from 5 L of culture broth)
15 were suspended in 100 ml of buffer and disrupted for 10 min using an ultrasonic
16 oscillator (Kubota, Osaka, Japan). This sample was ultracentrifuged at 40,000 rpm for
17 1 h (L-80 Ultracentrifuge; Beckman, Fullerton, CA, USA) and the supernatant was used
18 as a cell-free extract. The cell-free extract was fractionated with solid ammonium

1 sulfate. The precipitate at 30-50% saturation of ammonium sulfate was obtained by
2 centrifugation at 12,000 rpm for 20 min (High Mac CR21; Hitachi, Tokyo, Japan) and
3 dissolved in the same buffer. This enzyme solution (50 ml) was dialyzed against 3 L
4 of buffer for 12 h and the dialyzed enzyme solution was applied to a DEAE-Sephacel
5 column (3.0 × 7.0 cm; Amersham Pharmacia Biotech Co.) equilibrated with the same
6 buffer. After washing the column with 200 ml of buffer, the enzyme was eluted with a
7 linear gradient of 0-1.0 M NaCl in 250 ml buffer. The fractions containing the enzyme
8 activity were combined (270 ml) and dialyzed for 12 h against 3 L of buffer. After the
9 addition of 4 M NaCl, the enzyme solution was applied to a Phenyl-sepharose CL-4B
10 column (3.0 × 7.3 cm; Amersham Pharmacia Biotech Co.) equilibrated with buffer
11 containing 4 M NaCl. After washing the column with 500 ml of buffer, the enzyme
12 was eluted with a linear gradient of 0-0.5% (v/v) Tween 20 in 200 ml of buffer. The
13 fractions containing the enzyme activity were combined (125 ml) and dialyzed for 12 h
14 against 3 L of buffer comprising 20 mM Tris/HCl buffer (pH 9.0) and 0.2 mM PLP.
15 The dialyzed enzyme solution was concentrated to 10 ml by ultrafiltration using
16 Centriprep YM-3 (MILLIPORE, Bedford, MA, USA). The enzyme solution was
17 applied to a Mono Q HR 5/5 column (Amersham Pharmacia Biotech Co.) equilibrated
18 with buffer comprising 20 mM Tris/HCl buffer (pH 9.0) and 0.2 mM PLP. After

1 washing the column with the buffer, the enzyme was eluted with a linear gradient of
2 0-1.0 M NaCl in 60 ml of the buffer. The fractions containing the enzyme activity
3 were combined (4 ml) and dialyzed for 12 h against 2 L of buffer comprising 20 mM
4 potassium phosphate buffer (pH 7.0) and 0.2 mM PLP. This sample was used as
5 purified enzyme solution for characterization of its biochemical properties.

6 7 *2.7. Preparation of internal peptides from the purified enzyme*

8 Enzyme solution containing 1 mg of the purified enzyme was lyophilized and
9 dissolved in 100 μ l of buffer comprising 20 mM Tris/HCl (pH 9.0) and 8 M urea.
10 After incubation at 37°C for 60 min for denaturation, the concentration of urea was
11 decreased to 4 M by adding 100 μ l of 20 mM Tris/HCl buffer (pH 9.0). After the
12 addition of 0.05 nmol lysyl endopeptidase (Wako Pure Chemicals, Osaka, Japan), the
13 solution was incubated at 30°C for 6 h for digestion. The digested sample was applied
14 to a μ RPC C2/18 SC2/10 column (Amersham Pharmacia Biotech Co.) connected to a
15 SMART system (Amersham Pharmacia Biotech Co.) and equilibrated with mobile
16 phase of 0.1% (v/v) trifluoroacetic acid in water. The peptides were eluted using a
17 linear gradient of 0-80% (v/v) acetonitrile in 10 ml of mobile phase at a flow rate of 0.1
18 ml/min. The amino acid sequences of the internal peptides were analyzed by

1 automated Edman degradation with a 491HT protein sequencer (Applied Biosystems,
2 Foster City, CA, USA).

3 4 **3. Results**

5 *3.1. Purification of (S)-BAT*

6 (S)-BAT was purified about 17-fold by four successive steps from the cell-free extract
7 of *Variovorax* sp. JH2. Typical results of the purification are shown in Table 1. The
8 final preparation gave a single band on SDS-PAGE (Fig. 1) and showed specific activity
9 of 1.5 U/mg. The subunit molecular mass of purified enzyme determined by
10 SDS-PAGE was about 40,000 (Fig. 1). The native molecular mass determined by
11 gel-filtration chromatography was about 72,000.

12 13 *3.2. Kinetic parameters of (S)-BAT*

14 Kinetic parameters of (S)-BAT in the transamination reaction with racemic
15 β -phenylalanine and 2-oxoglutarate were investigated as described in Materials and
16 methods except that the reaction was performed with a variety of substrate
17 concentrations. The K_m value for racemic β -phenylalanine in the reaction with 10 mM
18 2-oxoglutarate was 0.63 mM, and the V_{max} value was 1.7 U/mg. The K_m value for

1
2 1 2-oxoglutarate in the reaction with 10 mM racemic β -phenylalanine was 2.5 mM.
3
4

5 2
6
7

8 3 *3.3. Stereoselectivity of (S)-BAT*
9

10
11 4 To investigate the stereoselectivity of (S)-BAT in the transamination of racemic
12

13
14 5 β -phenylalanine with 2-oxoglutarate for the enantioselective production of
15

16
17
18 6 (*R*)- β -phenylalanine from racemic β -phenylalanine, the time-course of the reaction was
19

20
21 7 analyzed essentially by the standard methods (Fig. 2). In a 1 h reaction, half of the 10
22

23
24 8 mM racemic β -phenylalanine was decomposed and about 5 mM benzoylactic acid was
25

26
27 9 produced. The optical purity of the remaining β -phenylalanine reached 99.6% *e.e.* for
28

29
30 10 (*R*)- β -phenylalanine. Longer incubation did not result in a further decrease in the
31

32
33
34 11 amount of β -phenylalanine. This result indicated that the purified enzyme catalyzed
35

36
37 12 (*S*)- β -phenylalanine-specific transamination. In the longer incubation, benzoylactic
38

39
40 13 acid was degraded gradually by spontaneous decarboxylation.
41

42
43 14
44
45

46 15 *3.4. Substrate specificity of (S)-BAT*
47

48
49 16 The substrate specificity of (S)-BAT was investigated (Table 2). As amino-donors,
50

51
52 17 10 mM β -phenylalanine analogs, some other β -amino acids, proteinogenic amino acids,
53

54
55
56 18 and their D-isomers were tested with 10 mM 2-oxoglutarate as an amino-acceptor.
57
58
59
60
61
62
63
64
65

1
2 1 Besides β -phenylalanine, 3-amino-3-(3-pyridyl)propionate served as a good substrate
3
4
5 2 for the enzyme and the relative activity was 56% of the enzymatic activity for racemic
6
7
8 3 β -phenylalanine. In the enantiomer analysis of the remaining amino acid during
9
10
11 4 racemic 3-amino-3-(3-pyridyl)propionate deamination, a decrease in only one
12
13
14 5 enantiomer was observed on HPLC analysis after GITC derivatization; indicating that
15
16
17
18 6 the enzyme had stereoselectivity for 3-amino-3-(3-pyridyl)propionate.

19
20
21 7 Extremely low activity was observed with L-glutamine, L-aspartate, L-asparagine,
22
23
24 8 β -alanine, and L-alanine, and the relative activities for these compounds were 1.1%,
25
26
27 9 0.57%, 0.30%, 0.28%, and 0.07% of the enzymatic activity for racemic β -phenylalanine,
28
29
30
31 10 respectively. The following compounds were judged to be inactive as amino-donors:
32
33
34 11 other proteinogenic amino acids (L-proline and L-glutamate were not tested), D-isomers
35
36
37 12 of 18 proteinogenic amino acids (D-proline and D-glutamate were not tested),
38
39
40 13 DL- β -aminoisobutyrate, taurine, L-phenylglycine, D-phenylglycine,
41
42
43 14 L-homophenylalanine, D-homophenylalanine, (*R*)-2-amino-2-phenylethanol,
44
45
46 15 (*S*)-2-amino-2-phenylethanol, (*R*)-1-phenylethylamine, (*S*)-1-phenylethylamine,
47
48
49 16 (*R*)-1-(1-naphthyl)ethylamine, (*S*)-1-(1-naphthyl)ethylamine, and,
50
51
52
53 17 1,2,3,4-tetrahydro-1-naphthylamine.

54
55
56 18 As an amino-acceptor, various oxo acids and ketones were tested at concentrations of
57
58
59
60
61
62
63
64
65

1 10 mM with 10 mM racemic β -phenylalanine as the amino-donor. Oxaloacetate,
2 pyruvate, and 1,3-acetonedicarboxylate served as the amino-acceptors. The relative
3 activities were 9.9%, 3.8%, and 2.0% of the enzymatic activity for 2-oxoglutarate,
4 respectively. The following compounds were judged to be inactive as
5 amino-acceptors: 3-phenylpyruvate, 2-oxovarelate, ketomalonate, 4-oxovalerate,
6 benzoylacetate, 2-heptanone, acetophenone, and ethyl benzoylacetate.

8 *3.5. Effects of pH and temperature on the activity and stability of the enzyme*

9 The optimum pH for the enzymatic activity (β -phenylalanine:2-oxoglutarate
10 transamination) was measured at 28°C under various pH conditions. The enzyme
11 showed a maximum activity at pH 9.5 (Fig. 3A). The optimum temperature for the
12 enzymatic activity was assayed at pH 9.0 at various temperatures (0-65°C). The
13 enzyme activity was found to be maximally active at 50°C (Fig. 3B).

14 The enzyme was incubated at 28°C for 30 min under various pH conditions, and then
15 a sample of the enzyme solution was taken and the residual activity
16 (β -phenylalanine:2-oxoglutarate transamination) was measured. The enzyme was
17 stable in the pH range 6.0-8.0 (Fig. 4A). The enzyme was preincubated for 30 min at
18 pH 9.0 under various temperatures (0-65°C) and the residual activity was assayed.

1 The enzyme was inactivated entirely by the incubation above 60°C (Fig. 4B).

2

3 *3.6. Effects of inhibitors and metal ions on the enzymatic activity*

4 The effects of various inhibitors and metal ions (2 mM) on the enzyme activity were
5 examined by the standard methods. The enzyme activity was inhibited by Ag⁺, Hg²⁺,
6 Zn²⁺, dinitrophenol, and hydroxylamine and decreased to 0%, 2.4%, 52%, 46%, and
7 23%, respectively, of the original activity. The other metal ions tested (Li⁺, Na⁺, K⁺,
8 Rb⁺, Cs⁺, Be²⁺, Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺, Fe²⁺, Co²⁺, Ni²⁺, Sn²⁺, Pb²⁺, Fe³⁺, Al³⁺, and Cr³⁺)
9 were judged not to affect the enzymatic activity. Metal ion chelaters
10 (ethylenediamine-*N,N,N',N'*-tetraacetic acid, 8-hydroxyquinoline, and
11 *o*-phenanthroline), serine protease inhibitors (phenylmethane sulfonylfluoride and
12 diisopropylphosphofluoride), and sulfhydryl inhibitors (5,5'-dithiobis-2-nitrobenzoate,
13 iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide) were judged to have no
14 significant influence on the activity.

15

16 *3.7. Analysis of amino acid sequences*

17 The amino acid sequences of internal peptides were analyzed and three parts of the
18 amino acid sequence were determined. These amino acid sequences were named IP1

1 (54 residues), IP2 (67 residues), and IP3 (63 residues) and were applied to homology
2 searches using BLAST (Fig. 5). All three amino acid sequences showed homology
3 with aminotransferases and, especially, β -aminotransferases and
4 glutamate-1-semialdehyde 2,1-aminomutases were most homologous with them
5 [20-23].

7 **4. Discussion**

8 As a result of the present investigation of substrate specificity, β -phenylalanine and
9 3-amino-3-(3-pyridyl)propionate were found to serve as good amino-donors for the
10 (*S*)-BAT-catalyzed reaction. The enzyme catalyzed transamination with the other
11 amino-donors such as L-glutamine, L-aspartate, L-asparagine, β -alanine, and L-alanine,
12 but the activities with these compounds were extremely low. These findings suggested
13 that the enzyme recognizes the 3-amino-3-aromatic propionate-skeleton specifically.
14 This result also indicated that the enzyme is different from already-known
15 ω -aminotransferases or aromatic aminotransferases. With regard to stereoselectivity
16 for amino-donors, the enzyme was found to catalyze (*S*)-enantiomer selective
17 transamination in the reactions with racemic β -phenylalanine and also showed
18 enantioselectivity in transamination with racemic 3-amino-3-(3-pyridyl)propionate.

1
2 1 Concerning the amino-acceptor, several oxo acids, such as 2-oxoglutarate, pyruvate,
3
4
5 2 oxaloacetate and 1,3-acetonedicarboxylate, served as substrates. In particular,
6
7
8 3 2-oxoglutarate was found to be the most suitable substrate as the amino-acceptor.
9
10
11 4 There are several differences between the (*S*)-BAT from *Variovorax* sp. JH2 and
12
13
14 5 β -aminotransferases from *Mesorhizobium* sp. in terms of substrate specificity. The
15
16
17 6 β -aminotransferases of *Mesorhizobium* sp. acted on some amino acids that are not the
18
19
20 7 substrate of (*S*)-BAT, such as β -alkyl- β -amino acids, aromatic α -amino acids
21
22
23 8 (phenylalanine and tyrosine), aliphatic α -amino acids (valine and leucine) and also acted
24
25
26 9 on arylamines, such as 1-phenylethylamine. Furthermore, both 2-oxoglutarate and
27
28
29 10 pyruvate have been reported to serve as good amino-acceptors for β -aminotransferases
30
31
32 11 from *Mesorhizobium* sp. These results indicated that β -aminotransferases from
33
34
35 12 *Mesorhizobium* sp. is an intermediate enzyme between β -aminotransferases,
36
37
38 13 ω -aminotransferases, and α -amino acid aminotransferases. These facts led to the
39
40
41 14 conclusion that the (*S*)-BAT from *Variovorax* sp. JH2 is a novel aminotransferase highly
42
43
44 15 specific to β -aryl- β -amino acids with preference for 2-oxoglutarate as an
45
46
47 16 amino-acceptor.
48
49
50

51
52
53 17 As the result of homology searches of the amino acid sequence, (*S*)-BAT showed the
54
55
56 18 highest homology with glutamate 1-semialdehyde 2,1-aminomutases as well as β -
57
58
59
60
61
62
63
64
65

1 aminotransferases [6]. These enzymes were classified into aminotransferase subgroup
2 II, containing acetylornithine, ornithine, ω -amino acid, 4-aminobutyric acid, and
3 diaminopelargonate aminotransferases, and it is possible that (*S*)-BAT is classified as a
4 member of the subgroup [24, 25]. It was reported that glutamate 1-semialdehyde
5 2,1-aminomutases act in porphyrin and chlorophyll metabolism in vivo, however,
6 (*S*)-BAT acts in β -phenylalanine assimilation of *Variovorax* sp. JH2 [25, 26]. In the
7 β -aminotransferases of *Mesorhizobium* sp., Arg43 and Arg404 supposed to be important
8 residues to recognize a carboxylate group [6]; however, the corresponding arginine
9 residues are not found in (*S*)-BAT (Fig. 5), which indicates the difference in these
10 enzymes.

11 Above all, it was clarified that (*S*)-BAT has a unique primary structure in
12 aminotransferase subgroup II and useful for optically active β -aryl- β -amino acid
13 production from racemic substrates by decomposition of specific enantiomer through
14 transamination in the presence of 2-oxoglutarate.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 **References**

- 2 [1] Achilles K, Schirmeister T, Otto HH. β -lactam derivatives as enzyme inhibitors:
3 1-peptidyl derivatives of 4-phenylazetidin-2-one as inhibitors of elastase and papain.
4 Arch Pharm Pharm Med Chem 2000;333:243-53.
- 5 [2] Nagano Y, Ikedo K, Fujishima A, Izawa M, Tsubotani S, Nishimura O, Fujino M.
6 Pyloricidins, novel anti-Helicobacter pylori antibiotics produced by *Bacillus* sp. J
7 Antibiotics2001;54:934-47.
- 8 [3] Roche D, Prasad K, Repic O. Enantioselective acylation of β -aminoesters using
9 penicillin G acylase in organic solvents. Tetrahedron Lett 1999;40:3665-8.
- 10 [4] Cohen SG, Weinstein SY. Hydrolysis of D(-)-ethyl β -phenyl- β -hydroxypropionate
11 and D(-)-ethyl β -phenyl- β -acetamidepropionate by α -chymotrypsin. J Am Chem Soc
12 1964;86:725-8.
- 13 [5] Faulconbridge SJ, Holt KE, Sevillano LG, Lock CJ, Tiffin PD, Tremayne N, Winter
14 S. Preparation of enantiomerically enriched aromatic β -amino acids via enzymatic
15 resolution. Tetrahedron Lett 2000;41:2679-81.
- 16 [6] Kim J, Kyung D, Yun H, Cho BK, Seo JH, Cha M, Kim BG. Cloning and
17 characterization of a novel β -aminotransferases from *Mesorhizobium* sp. Strain LUK: a
18 new biocatalyst for the synthesis of enantiomerically pure β -amino acids. Appl Environ

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 Microbiol 2007;73:1772-82.

2 [7] Kim J, Kyung D, Yun H, Cho B, Kim BG. Screening and purification of a novel
3 aminotransferases catalyzing the transamination of aryl β -amino acid from
4 *Mesorhizobium* sp. LUK. J Microbiol Biothechnol 2006;16:1832-6.

5 [8] Mano J, Ogawa J, Shimizu S. Microbial production of optically active
6 β -phenylalanine through stereoselective degradation of racemic β -phenylalanine. Biosci
7 Biotechnol Biochem 2006;70:1941-6.

8 [9] Yonaha K, Suzuki K, Toyama S. *Streptomyces* β -Alanine: α -ketoglutarate
9 aminotransferase, a novel ω -aminoacid transferase. J. Biol. Chem., 260, 3265-3268
10 (1985)

11 [10] Toyama S, Misono H, Soda K. Properties of taurine:alpha-ketoglutarate
12 aminotransferase of *Achromobacter superficialis*. Inactivation and reactivation of the
13 enzyme. Biochem Biophys Acta1978;14:75-81.

14 [11] Arst HN Jr. GABA transaminase provides an alternative route of beta-alanine
15 synthesis in *Aspergillus nidulans*. Mol Gen Genet 1978;6:23-7.

16 [12] Yonaha K, Nishie M, Aibara S. The primary structure of omega-amino
17 acid:pyruvate aminotransferase. J Biol Chem 1992;25:12506-10.

18 [13] Garabedian PA. *Candida* δ -aminovalerate: α -ketoglutarate aminotransferase:

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 purification and enzymologic properties. *Biochemistry* 1986;23:5507-12.

2 [14] Poston JM. Leucine 2,3-aminomutase, an enzyme of leucine catabolism. *J Biol*
3 *Chem* 1976;251:1859-63.

4 [15] Kinoshita T, Kasahara Y, Nimura N. Reversed-phase high-performance liquid
5 chromatographic resolution of non-esterified enantiomeric amino acids by derivatization
6 with 2,3,4,6-tetra-o-acetyl- β -D-glucopyranosyl isothiocyanate and
7 2,3,4-tri-o-acetyl- α -D-arabinopyranosyl isothiocyanate. *J Chromatogr* 1981;210:77-81.

8 [16] Nimura N, Ogura H, Kinoshita T. Reversed-phase liquid chromatographic
9 resolution of amino acid enantiomers by derivatization with
10 2,3,4,6-tetra-o-acetyl- β -D-glucopyranosyl isothiocyanate. *J Chromatogr*
11 1980;202:375-9.

12 [17] Nimura N, Toyama A, Kinoshita T. Optical resolution of amino acid enantiomers
13 by high-performance liquid chromatography. *J Chromatogr* 1984;316:547-52.

14 [18] Bradford MM. A rapid sensitive method for the quantification of microgram
15 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*
16 1972;72:248-54.

17 [19] King J, Laemmli U. Polypeptides of the tail fibres of bacteriophage T4. *J Mol Biol*
18 1971;62:465-77.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 [20] Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A, et al. The
2 genome sequence of *Schizosaccharomyces pombe*. Nature 2002;415:871-80.

3 [21] Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N, et al. Complete
4 genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic
5 sequence comparison with *Bacillus subtilis*. Nucleic Acids Res 2000;28:4317-31.

6 [22] Cerdeno-Tarraga AM, Efstratiou A, Dover LG, Holden MTG, Pallen M, Bentley SD,
7 et al. The complete genome sequence and analysis of *Corynebacterium diphtheriae*
8 NCTC13129. Nucleic Acids Res 2003;31:6516-23.

9 [23] Loftus BJ, Fung E, Roncaglia P, Rowley D, Amedeo P, Bruno D, et al. The
10 Genome of the Basidiomycetous Yeast and Human Pathogen *Cryptococcus neoformans*.
11 Science2005;25:1321-4.

12 [24] Mehta PK, Hale TI, Christen P. Aminotransferases: demonstration of homology and
13 division into evolutionary subgroups. Eur J Biochem 1993;214:549-61.

14 [25] D'Aguanno S, Gonzales IN, Simmaco M, Contestabile R, John RA.
15 Stereochemistry of the reactions of Glutamate-1-semialdehyde aminomutase with
16 4,5-diaminovalerate. J Bio Chem 2003;278:40521-6.

17 [26] Hennig M, Grimm B, Contestabile R, John RA, Jansonius JN. Crystal structure of
18 glutamate-1-semialdehyde aminomutase: an α_2 -dimeric vitamin B₆-dependent enzyme

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 with asymmetry in structure and active site reactivity. Proc Natl Acad Sci USA
2
3
4
5 2 1997;94:4866-71.
6
7
8 3
9

1
2 **1 Figure legends**
3
4

5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2
3 **Fig. 1** SDS-PAGE of the purified enzyme. Lane A is 1 mg of the purified enzyme.

4 Lane B is molecular mass standards (from top): phosphorylase *b* (97 kDa), BSA (66
5 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and
6 lysozyme (14 kDa).
7

8 **Fig. 2** Time-course of the transamination with purified (*S*)-BAT. Concentrations of
9 β -phenylalanine (BPA) (\square) and benzoylactic acid (\bullet) are shown. Optical purity of
10 β -phenylalanine in the 1 h reaction is shown with an arrow.
11

12 **Fig. 3** Effects of pH and temperature on the activity of (*S*)-BAT. The effects of pH
13 (A) and temperature (B) on the enzymatic activity (β -phenylalanine:2-oxoglutarate
14 transamination) are shown. Buffers (200 mM) used for pH adjustment are as follows:
15 acetate/sodium acetate buffer (\square) for pH 5.0-6.0, potassium phosphate buffer (\blacktriangle) for
16 pH 6.0-8.0, Tris/HCl buffer (\bullet) for pH 7.5-10.0, borate/NaOH buffer (\circ) for pH
17 10.0-10.5, $\text{Na}_2\text{PO}_4/\text{NaOH}$ buffer (\blacksquare) for pH 10.5-12.0.
18

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 **Fig. 4** Effects of pH and temperature on the stability of (S)-BAT. The effects of pH
2 (A) and temperature (B) on the stability are shown. Buffers (0.5 M) used for pH
3 adjustment are as follows: acetate/sodium acetate buffer (□) for pH 4.0-6.0, potassium
4 phosphate buffer (▲) for pH 6.0-8.0, Tris/HCl buffer (●) for pH 8.0-10.0, borate/NaOH
5 buffer (○) for pH 9.0-11.0, Na₂PO₄/NaOH buffer (■) for pH 11.0-12.0.

7 **Fig. 5** Amino acid sequence alignments of IP1-3 and proteins homologous to them.
8 Identical amino acids to those in the sequences of IP1-3 are marked in gray. MES, β-
9 aminotransferases from *Mesorhizobium* sp.; CNE, glutamate-1-semialdehyde
10 2,1-aminomutase from *Cryptococcus neoformans*; SPO, glutamate-1-semialdehyde
11 2,1-aminomutase from *Schizosaccharomyces pombe*; BHA, glutamate-1-semialdehyde
12 2,1-aminomutase from *Bacillus halodurans*; FRA, aminotransferase class-III from
13 *Frankia* sp.; CDI, glutamate-1-semialdehyde 2,1-aminomutase from *Corynebacterium*
14 *diphtheriae*.

Figure1
[Click here to download high resolution image](#)

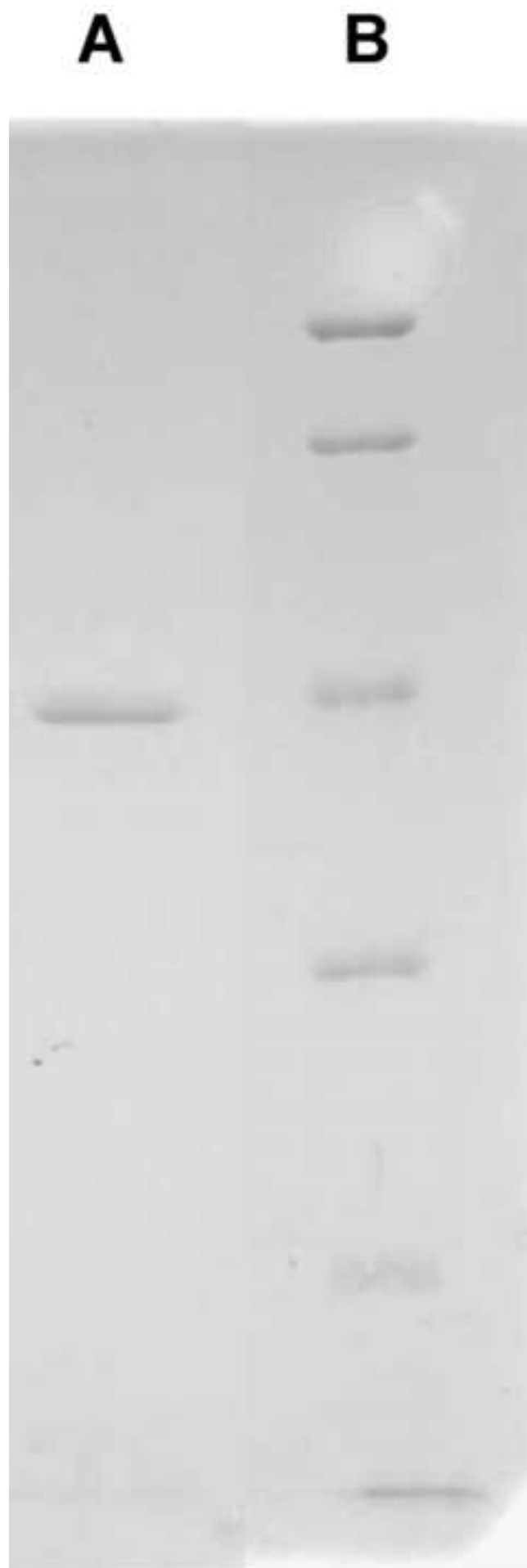


Figure2
[Click here to download high resolution image](#)

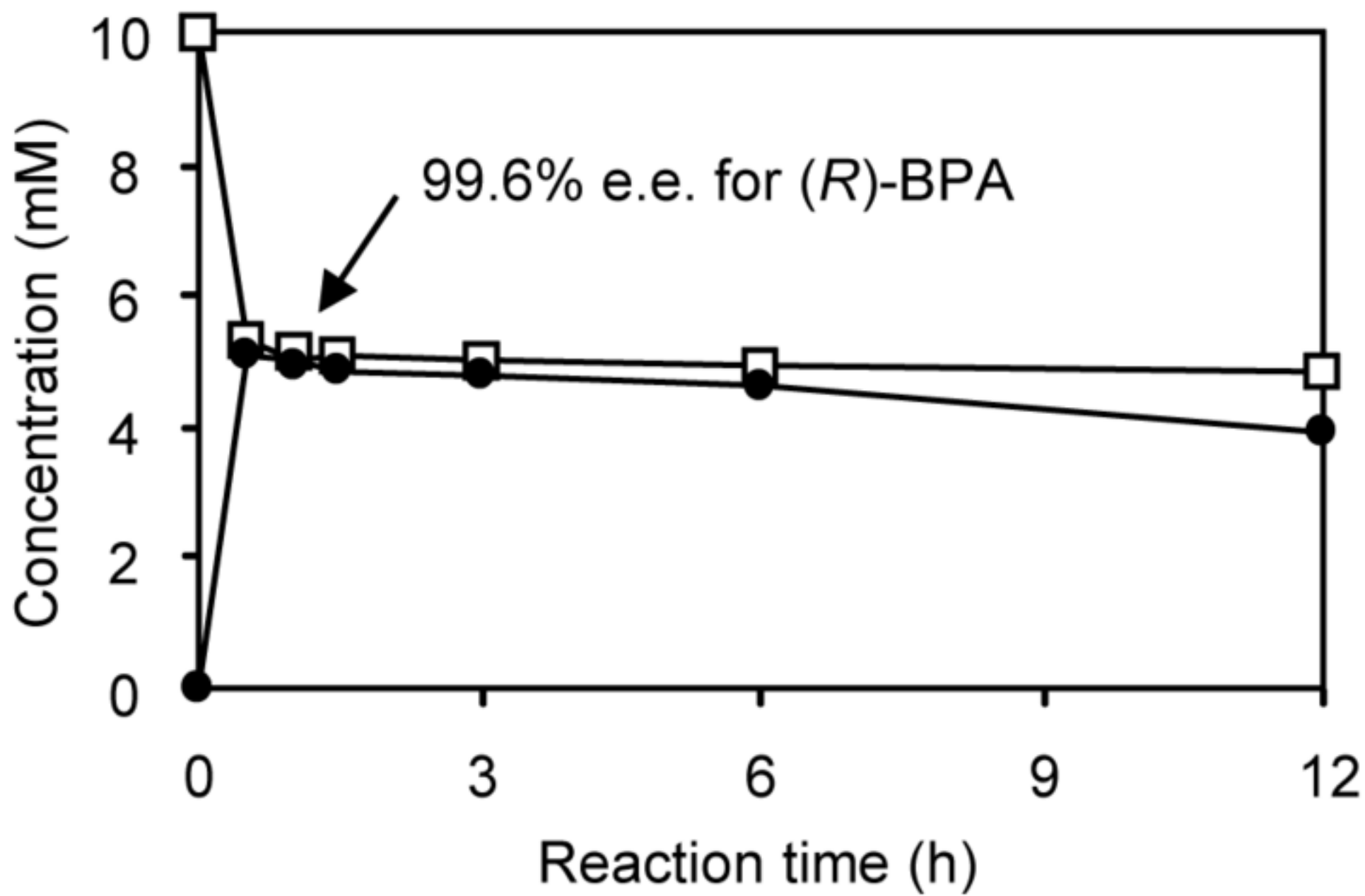


Figure3
[Click here to download high resolution image](#)

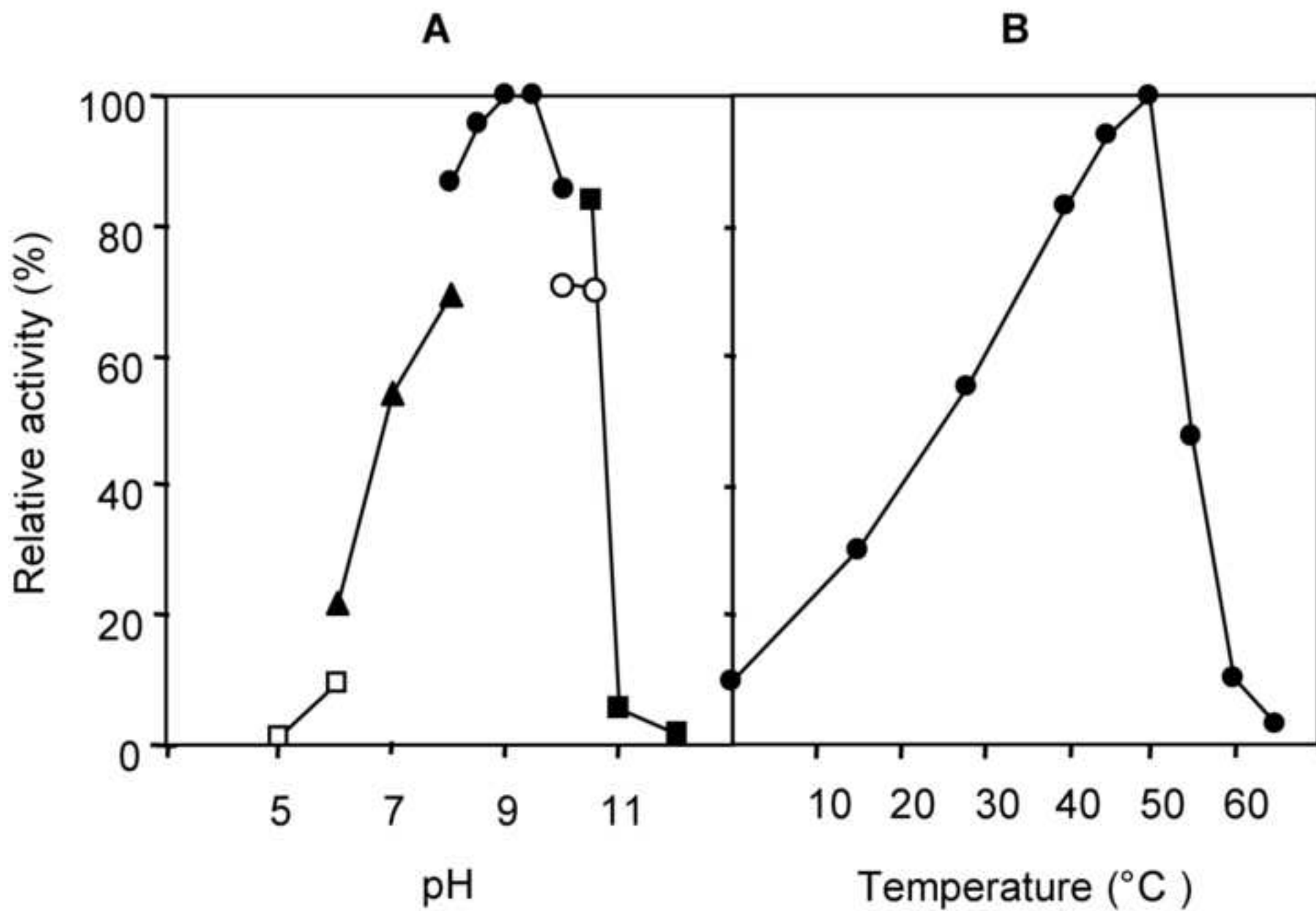


Figure4
[Click here to download high resolution image](#)

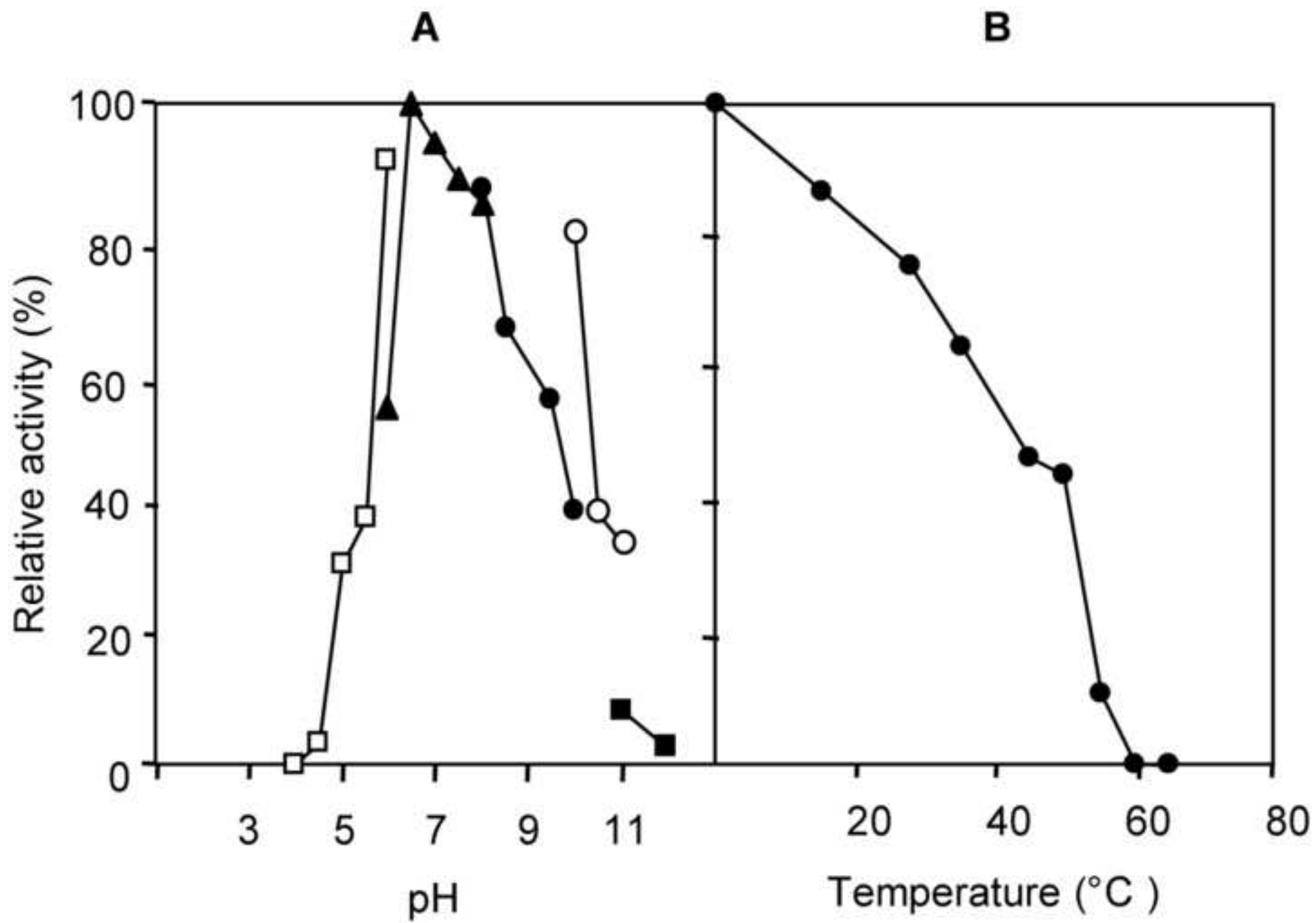


Figure 5

[Click here to download high resolution image](#)

A

IP1	1	T L A P I D H A L A E A H R R F T E A N P A S R R Q F E A N A E S M P G G N T R	40
MES	15	D T A E K A Q A I A A A R N T F A R D N P V S A G H H E R A R R S M P G G N T R	54
CNE	-1	- M T S L T Q A L K R A Q Q T Y A N R N R K S Y E A H Q A A S R H L P G G S T R	39
SPO	4	E I S D L S L A K E K S E A Q F I A R N P K S N D F H C K A C D S L P G G N T R	44
BHA	-3	- - - M A M V E D T R P E H T Y A E Q T P R S K S W I E Q A K K V M P G G I T A	37
IP1	41	S V L F Y A P P P L T I A R	54
MES	55	S I L F H R P P P L V I A Q	68
CNE	40	S S I F I H P P P L C I Q K	53
SPO	45	T V L H G A P P P I F I E A	58
BHA	38	N I K H F A F Y F I V M K K	51

B

IP2	1	Y I G G G M S F G A F G G R A D V M A Q F D P R T - E A L A H S G T E F N N N V M	39
MES	281	Y I G G G M S F G A F G G R R D L M E R F D P A R D G A F A H A G T F N N N I L	320
SPO	271	Y L G G G L P P F G A F G G R A D I M S C F D P R L P G S L S H S G T F N N D T L	310
CNE	269	F F G G G F A F G A F G G K K E I M E K F D A R K G G A I S H G G T F N N S P L	308
FRA	296	A L G G G L P S G A I G A T A E L M E A V A S D R - - - V K Q V G T Y N G N P L	332
IP2	40	T M A A G Q A G L T R L F T P E A A G X L X E R G X A M	67
MES	321	T M S A G H A A L T Q I Y T R Q A A S D L S A S G D R F	348
SPO	311	T L T A G Y V G L T E L Y T P E A V K R L N A L G D G L	338
CNE	309	T M V A G A T A M E K I L T E D A L K N L N K L G D W M	336
FRA	333	T M A A A R A S L F E V L T P D A Y T H L D R L G G R L	360

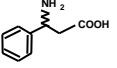
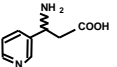
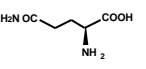
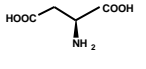
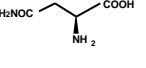


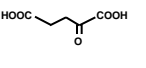
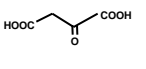

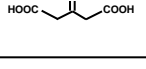
C

IP3	1	E G V A L Q F T G V G S L M N A H F L Q - G E V R R V A D L A A V D G R L R Q L	39
MES	359	N O A P L Q F T G L G S L G T I H F S R - A P I R S A G D V R A A D Q Q L K E L	397
BHA	377	K G V P M K A V G I G S I F S V V L T E - E D I F N Y R D L O A S D I Q T R K L	415
SPO	349	- - T K M S I T G L G S I M N I H F T E S G R V N S Y N D T A G E V I E L K D L	386
CDI	343	E G V A H H I Q R A A T M L S V R F A E - G E G H N F D D M K A A D T P R P P A	381
IP3	40	L F F H L L N A G I Y T S P R G F V V L S L P P	63
MES	398	F F F H M L R K G I Y L A P R G M Y A L S L E I	421
BHA	416	I D L H L L N E G I Y T K P - - L N R Y S L S T	437
SPO	387	L W M D L L K E G F W I A R R G M I T L S L V L	410
CDI	382	F F H A L L D N G I Y A S P S V F E T W P V S A	405

Table 1. Purification of (*S*)-BAT

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	1260	109	0.087	100
Ammonium sulfate fractionation	410	83	0.20	76
DEAE sephacel	170	42	0.24	38
Phenyl sepharose	4.0	3.4	0.85	3.1
Mono Q HR 5/5	0.1	0.14	1.5	0.13

Table 2. Substrate specificity of (*S*)-BAT for amino donor (A) and for amino acceptor (B)

(A)		
Amino donor	Structural formula	Relative activity (%)
Racemic BPA		100
Racemic 3-amino-3-(3-pyridyl)propionic acid		56.0
L-Glutamine		1.1
L-Aspartate		0.57
L-Asparagine		0.30
β -Alanine		0.28
L-Alanine		0.07
(B)		
Amino acceptor	Structural formula	Relative activity (%)
2-Oxoglutarate		100
Oxaloacetate		9.9
Pyruvate		3.8
1,3-Acetonedicarboxylate		2.0