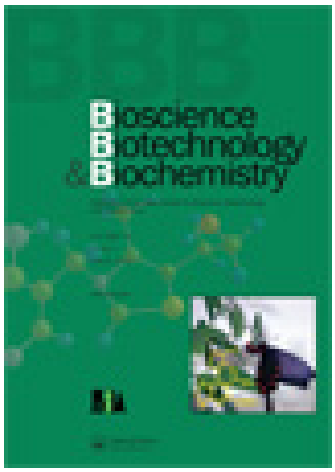


Bioscience, Biotechnology, and Biochemistry

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

A novel S-enantioselective amidase acting on 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide from Arthrobacter sp. S-2

Ken-ichi Fuhshuku[a], Shunsuke Watanabe[a], Tetsuro Nishii[c], Akihiro Ishii[c] & Yasuhisa Asano[ab]

[a] Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Japan

[b] Asano Active Enzyme Molecule Project, ERATO, JST, Imizu, Japan

[c] Chemical Research Center, Central Glass Co., Ltd., Kawagoe, Japan

Published online: 26 May 2015.

To cite this article: Ken-ichi Fuhshuku, Shunsuke Watanabe, Tetsuro Nishii, Akihiro Ishii & Yasuhisa Asano (2015): A novel S-enantioselective amidase acting on 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide from Arthrobacter sp. S-2, Bioscience, Biotechnology, and Biochemistry, DOI: 10.1080/09168451.2015.1038216

To link to this article: http://dx.doi.org/10.1080/09168451.2015.1038216

Bioscience, Biotechnology, and Biochemistry, 2015

A novel *S*-enantioselective amidase acting on 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide from *Arthrobacter* sp. S-2

Ken-ichi Fuhshuku[1], Shunsuke Watanabe[1], Tetsuro Nishii[3], Akihiro Ishii[3] and Yasuhisa Asano[1,2,*]

[1]*Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Japan;* [2]*Asano Active Enzyme Molecule Project, ERATO, JST, Imizu, Japan;* [3]*Chemical Research Center, Central Glass Co., Ltd., Kawagoe, Japan*

Received February 20, 2015; accepted March 20, 2015
http://dx.doi.org/10.1080/09168451.2015.1038216

A novel *S*-enantioselective amidase acting on 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide was purified from *Arthrobacter* sp. S-2. The enzyme acted *S*-enantioselectively on 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide to yield (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid. Based on the N-terminal amino acid sequence of this amidase, the gene coding *S*-amidase was cloned from the genomic DNA of *Arthrobacter* sp. S-2 and expressed in an *Escherichia coli* host. The recombinant *S*-amidase was purified and characterized. Furthermore, the purified recombinant *S*-amidase with the C-His_6-tag, which was expressed in *E. coli* as the C-His_6-tag fusion protein, was used in the kinetic resolution of (±)-3,3,3-trifluoro-2-hydroxy-2-methylpropanamide to obtain (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid and (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanamide.

Key words: amidase; *Arthrobacter* sp.; 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid; 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide

The synthesis of enantiomerically pure trifluoromethyl-substituted compounds has recently being attracting the attention of scientists for its wide applications such as pharmaceutical and agrochemical intermediates.[1)] Of these, both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid **1** were previously shown to be potent intermediates for the synthesis of a number of fine chemicals and pharmaceuticals, such as a pyruvate dehydrogenase kinase inhibitor and bradykinin antagonist (Fig. 1).[2,3)]

In order to prepare carboxylic acids, the hydrolysis of their corresponding amides is utilized as the most traditional method. However, amides sometimes have to be hydrolyzed under harsh conditions using chemical methods. On the other hand, enzymatic and biocatalytic methods can produce carboxylic acids by the hydrolysis of their corresponding amides under mild conditions, and have increased in importance in the last few decades.[4)] Although few studies have examined the hydrolysis of trifluoromethyl-substituted aliphatic amides, Shaw developed a process for the large-scale synthesis of enantiomerically pure acid **1** by a kinetic resolution using the *R*-enantioselective amidase from *Klebsiella oxytoca* PRS1.[5,6)]

In our previous study, the enantioselective hydrolysis activity acting on amide **2** was screened from microbial sources, and *Shinella* sp. R-6 and *Arthrobacter* sp. S-2, which exhibited *R*- and *S*-enantioselective hydrolysis activities toward amide **2**, respectively, were isolated.[7)] Furthermore, the preparation of both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid via the two-step whole-cell reaction was investigated using these two strains.

In this study, we described the purification of *S*-amidase from *Arthrobacter* sp. S-2 that *S*-enantioselectively acts on amide **2**. The gene coding this *S*-amidase was cloned and expressed in an *Escherichia coli* host. The recombinant *S*-amidase was purified and characterized. Furthermore, the purified recombinant *S*-amidase with the C-His_6-tag, which was expressed in *E. coli* as the C-His_6-tag fusion protein, was used in the kinetic resolution of (±)-amide **2** to obtain (*S*)-acid **1** and (*R*)-amide **2** (Scheme 1).

Materials and methods

Materials. (±)-Amide **2** was kindly gifted from Central Glass Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from commercial sources and used without further purification.

Enzyme assay and definition of units. Enzyme activity was assayed by measuring the conversion of amide **2** to acid **1**. The enzyme solution was added to 20 mM potassium phosphate buffer (KPB, pH 7.0), containing 10 μmol of (±)-amide **2** in a total volume

*Corresponding author. Email: asano@pu-toyama.ac.jp

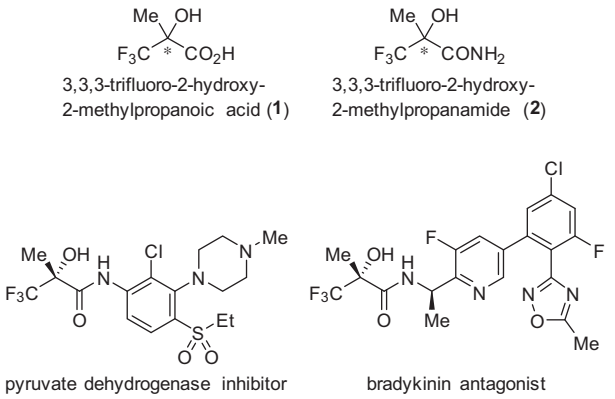


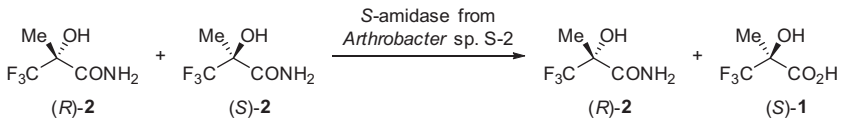
Fig. 1. 3,3,3-Trifluoro-2-hydroxy-2-methylpropanoic acid **1** and its corresponding amide **2**, as intermediates for the synthesis of pharmaceuticals.

of 200 µL. After being incubated for an appropriate period at 30 °C, the reaction mixture was mixed with 50 µL of 0.1 N HCl. After centrifugation (20,000 × *g*, 10 min, 4 °C), a 600-µL aliquot was withdrawn and mixed with 400 µL of water. After centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatant was analyzed using HPLC with a Cosmosil® C_{18}-MS-II column (5 µm, 4.6 mm × 150 mm, Nacalai Tesque, Inc., Kyoto, Japan) (solvent: 0.1% H_3PO_4 aq./MeCN = 9/1; flow rate: 0.80 mL/min; temperature: 40 °C; detection: 210 nm; t_R = 4.7 min for amide **2** and 7.6 min for acid **1**) for the conversion and with a Sumichiral OA-5000 column (5 µm, 4.6 mm × 150 mm, Sumika Chemical Analysis Service, Ltd.) [solvent: 2 mM $CuSO_4$ aq./MeCN = 85/15; flow rate: 2.0 mL/min; temperature: 40 °C; detection: 254 nm; t_R = 13.6 min for (*R*)-**1** and 16.0 min for (*S*)-**1**] for the enantiomeric excess. One unit of enzyme activity was defined as the amount of enzyme that converted 1 µmol of amide **2** to acid **1** per min under standard assay conditions.

Protein measurements. Protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard.[8)]

Analytical methods. Native- and SDS-PAGE were carried out as described by Davis[9)] and Laemmli,[10)] respectively, with an electrophoresis unit (ATTO Corp., Tokyo, Japan). The molecular mass of a subunit of the enzyme was estimated by SDS-PAGE on the basis of mobility relative to those of standard proteins (Bio-Rad Laboratories, Hercules, CA, USA): phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The protein bands of SDS-PAGE were stained with Brilliant blue G (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and destained in MeOH/AcOH/water (3/1/6). Activity staining of Native-PAGE was carried out as described below on the basis of the method by Fournand et al.[11)] The gel after Native-PAGE was immersed in 10 mL of 20 mM KPB (pH 7.0), containing 500 µmol of (±)-amide **2** and 1.0 mmol of NH_2OH. After being incubated for 1 min at 30 °C, 20 mL of 355 mM $FeCl_3$ in 0.65 N HCl solution was added to the mixture. The molecular mass of the native enzyme was estimated by gel-filtration HPLC with a Superdex 200 10/300 GL (GE Healthcare UK Ltd., Buckinghamshire, England) column with 20 mM KPB (pH 7.0), containing 150 mM NaCl as the eluent at 0.45 mL/min on the basis of mobility relative to those of standard proteins (Oriental Yeast Co., Ltd., Tokyo, Japan): glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome C (12.4 kDa).

Purification of S-*amidase from* Arthrobacter *sp. S-2.* A cultivation medium consisting of 5.0 g of glycerol, 5.0 g of $(NH_4)H_2PO_4$, 2.0 g of KH_2PO_4, 2.0 g of K_2HPO_4, 1.0 g of NaCl, 0.50 g of yeast extract, 0.20 g of $MgSO_4$, 1.0 mL of the vitamin mixture solution,[7)] and 1.0 mL of the trace element solution[7)] in 1,000 mL of water was used for the cultivation of *Arthrobacter* sp. S-2 cells. *Arthrobacter* sp. S-2 cells were inoculated into 5.0 mL of the cultivation medium described above and incubated with reciprocal shaking at 300 strokes/min for 24 h at 30 °C. Grown cells were added to 500 mL of the same medium and incubated with shaking at 150 rpm for 24 h at 30 °C. Cells were harvested by centrifugation (9,000 × *g*, 10 min, 4 °C) and washed with 20 mM KPB (pH 7.0). Unless otherwise stated, all purification procedures were performed at below 4 °C, and KPB (pH 7.0) containing 5 mM 2-mercaptoethanol and 20% glycerol was used as the buffer throughout this purification. Wet cells (ca. 600 g) from 50 L of the culture were suspended in 3 L of 20 mM buffer and then disrupted by Insonator 201 M (19 kHz, Kubota Corp., Tokyo, Japan) for 30 min at 4 °C. After centrifugation (20,000 × *g*, 15 min, 4 °C), the supernatant was used as the cell-free extract. The cell-free extract was applied to a Q Sepharose (GE Healthcare UK Ltd., Buckinghamshire, England) column (400 mL) equilibrated with 20 mM buffer. After the column had been washed thoroughly with 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–300 mM) in 20 mM buffer. The combined active fractions were then brought to 30% $(NH_4)_2SO_4$ saturation and applied to a Butyl-Toyopearl® 650M (Tosoh Corp., Tokyo, Japan) column (100 mL) equilibrated with 20 mM buffer containing 30% saturated $(NH_4)_2SO_4$. After the column had been washed thoroughly with 20 mM buffer containing 30%



Scheme 1. Enantioselective hydrolysis of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide **2** by *S*-amidase from *Arthrobacter* sp. S-2.

saturated $(NH_4)_2SO_4$, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ (30–0%) in 20 mM buffer. The combined active fractions were dialyzed with 1.0 mM buffer and applied to a Gigapite (Seikagakukogyo Corp., Tokyo, Japan) column (100 mL) equilibrated with 1.0 mM buffer, and the flow-through was collected. This flow-through was applied to a DEAE-Toyopearl® 650M (Tosoh Corp., Tokyo, Japan) column (20 mL) equilibrated with 20 mM buffer. After the column had been washed thoroughly with 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–300 mM) in 20 mM buffer. The combined active fractions were then brought to 30% $(NH_4)_2SO_4$ saturation and applied to a Resource PHE (GE Healthcare UK Ltd., Buckinghamshire, England) column (1 mL) equilibrated with 20 mM buffer containing 30% saturated $(NH_4)_2SO_4$. After the column had been washed thoroughly with 20 mM buffer containing 30% saturated $(NH_4)_2SO_4$, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ (30–0%) in 20 mM buffer using the ÄKTA explorer 10S (GE Healthcare UK Ltd., Buckinghamshire, England) at 0.20 mL/min. The combined active fractions were dialyzed with 20 mM buffer, concentrated by Amicon® Ultra-4 (Merck KGaA, Darmstadt, Germany), and applied to a Superdex 200 HR 10/30 (GE Healthcare UK Ltd., Buckinghamshire, England) column (24 mL) equilibrated with 20 mM buffer. The enzyme was eluted with 20 mM buffer using the ÄKTA explorer 10S at 0.20 mL/min. The active fraction was used to analyze N-terminal amino acid sequence of the enzyme, and its analysis was performed at APRO Life Science Institute Inc. (Tokushima, Japan) with a Procise 494 HT protein sequence system.

Cloning of the gene coding S-*amidase from* Arthrobacter *sp. S-2.* Genomic DNA was prepared from *Arthrobacter* sp. S-2 cells by the method of Misawa[12] and used as a template for PCR. A partial gene coding *S*-amidase from *Arthrobacter* sp. S-2 was amplified using genomic DNA as a template with two combinations of primer F1 (5′-ATGCARGGNYTNCCNACNMG-3′) and cassette primer C1 (5′-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3′), or primer F2 (5′-WSNAAYGGNGCNATH-3′) and cassette primer C2 (5′-CGTTAGAACGCGTAATACGACTCACTATAGGGAGA-3′) using the LA PCR™ *in vitro* cloning kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The sequences of F1 and F2 were designed based on the N-terminal amino acid sequence. The two cassette primers C1 and C2 corresponded to the cassette in the LA PCR™ *in vitro* cloning kit. The first PCR was carried out as follows. After initial denaturation of genomic DNA for 2 min 30 s at 94 °C, amplification was performed in 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 2 min at 72 °C in 50 μL of the reaction mixture containing 100 ng of genomic DNA, 0.20 μM each of the F1 and C1 primers, 0.40 mM dNTPs, 1× LA Buffer II (Mg^{2+} plus), and 2.5 U of TaKaRa LA Taq® DNA polymerase (Takara Bio Inc., Shiga, Japan). The second PCR was carried out using 50 ng of the first PCR product as a template under the same conditions as the first PCR with the primers F2 and C2. The two PCRs yielded a product that was approximately 1.2 kb, which was cloned into the Novagen® pT7 Blue T-vector (Merck KGaA, Darmstadt, Germany) and sequenced. To obtain the complete sequence of the gene coding *S*-amidase, inverse PCR was carried out as follows. Genomic DNA was digested with some appropriate restriction enzymes, incubated for 24 h at 37 °C, and then circularized by T4 DNA ligase (Takara Bio Inc., Shiga, Japan) for 1 h at 16 °C. The primers InF (5′-CATCGTTCCGGTAGGTGAAGCCGTTC-3′) and InR (5′-GCAGCAACAGGAACGTCTGCCCTCATC-3′) were designed based on the partial gene sequenced above. Inverse PCR was carried out under the same PCR conditions described above. The PCR product was cloned into the Novagen® pT7 Blue T-vector and sequenced. The gene sequence of *S*-amidase from *Arthrobacter* sp. S-2 has been deposited into GenBank with the accession number LC026945.

Construction of a plasmid and transformation of E. coli *for the expression of the gene coding* S-*amidase from* Arthrobacter *sp. S-2.* *Nde*I-*Xho*I fragments containing the gene coding *S*-amidase were amplified with the primers Aas-F (5′-GATACATATGCAGGGACTGCCAACACGG-3′) and Aas-R (5′-GATACTCGAGTCAGACCGTCCGGCTTGATC-3′), and Aas-F and AasH-R (5′-GATACTCGAGGACCGTCCGGCTTGATC-3′, for the C-terminal His_6-tag) including appropriate restriction sites (underlined sequence), and genomic DNA as a template. Each of the amplified PCR products was digested with *Nde*I and *Xho*I, and was ligated into the expression vector Novagen® pET23a(+) or pET15b (Merck KgaA, Darmstadt, Germany) digested with the same restriction enzymes, respectively. Each of the recombinant plasmids pET23a(+)-*S*-amidase, pET23a(+)-*S*-amidase with the C-His_6-tag, and pET15b-*S*-amidase, named pEAAS23, pEAAS23-CH, and pEAAS15, respectively, was used to transform *E. coli* BL21(DE3) or *E. coli* BL21-CodonPlus®(DE3)-RIPL (Agilent Technologies Inc., Santa Clara, CA, USA), respectively.

Production and purification of recombinant S-*amidase in* E. coli *BL21-CodonPlus®(DE3)-RIPL transformants.* *E. coli* BL21-CodonPlus®(DE3)-RIPL harboring pEAAS23 was inoculated into 5.0 mL of Luria–Bertani (LB) medium consisting of 10 g of Hipolypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 5.0 g of yeast extract, and 10 g of NaCl in 1000 mL of water containing 80 μg/mL of ampicillin and 50 μg/mL chloramphenicol and then incubated with reciprocal shaking at 300 strokes/min for 12 h at 37 °C. The cells that grew were added to 500 mL of the same medium and incubated with shaking at 150 rpm for 12 h at 37 °C. When the optical density at 610 nm of the medium reached 0.5–1.0, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and the culture was further incubated for 10 h at 30 °C. Cells were harvested by centrifugation (6000 × *g*, 10 min, 4 °C) and washed with 20 mM KPB (pH 7.0). Unless otherwise stated,

all purification procedures were performed below 4 °C, and KPB (pH 7.0), containing 5 mM 2-mercaptoethanol and 20% glycerol, was used as the buffer throughout the purification. Wet cells (ca. 10 g) from 2.0 L of the culture were suspended in 50 mL of 20 mM buffer and then disrupted by Insonator 201M (19 kHz) for 30 min at 4 °C. After centrifugation (20,000 × *g*, 15 min, 4 °C), the supernatant was used as the cell-free extract. The cell-free extract was applied to a Q Sepharose column (50 mL) equilibrated with 20 mM buffer. After the column had been washed thoroughly with 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–300 mM) in 20 mM buffer. The combined active fractions were then brought to 30% $(NH_4)_2SO_4$ saturation and applied to a Butyl-Toyopearl® 650M column (10 mL) equilibrated with 20 mM buffer containing 30% saturated $(NH_4)_2SO_4$. After the column had been washed thoroughly with 20 mM buffer containing 30% saturated $(NH_4)_2SO_4$, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ (30–0%) in 20 mM buffer. The combined active fractions were dialyzed with 20 mM buffer and applied to a Mono Q HR 5/5 column (1 mL) equilibrated with 20 mM buffer. After the column had been washed thoroughly with 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–300 mM) in 20 mM buffer using the ÄKTA explorer 10S at 0.20 mL/min. The combined active fractions were concentrated by Amicon® Ultra-4, applied to a Superdex 200 HR 10/30 column (24 mL) equilibrated with 20 mM buffer, and eluted with 20 mM buffer using the ÄKTA explorer 10S at 0.20 mL/min. The combined active fractions were collected and used for the characterization of *S*-amidase from *Arthrobacter* sp. S-2.

Production and purification of recombinant S*-amidase with the C-His$_6$-tag in* E. coli *BL21-CodonPlus®(DE3)-RIPL transformants.* The cultivation of *E. coli* BL21-CodonPlus®(DE3)-RIPL harboring pEAAS23-CH was carried out similar to that of *E. coli* BL21-CodonPlus®(DE3)-RIPL harboring pEAAS23, as described above. Unless otherwise stated, all purification procedures were performed below 4 °C, and KPB (pH 7.0), containing 5 mM 2-mercaptoethanol and 20% glycerol, was used as the buffer throughout the purification. Wet cells (ca. 10 g) from 2 L of the culture were suspended in 50 mL of 20 mM buffer containing 300 mM NaCl and 20 mM imidazole, and were then disrupted as described above. After centrifugation (20,000 × *g*, 15 min, 4 °C), the supernatant was used as the cell-free extract. The cell-free extract was applied to a Ni Sepharose 6 Fast Flow (GE Healthcare UK Ltd., Buckinghamshire, England) column (5.0 mL) equilibrated with 20 mM buffer containing 300 mM NaCl and 20 mM imidazole. After the column had been washed thoroughly with 20 mM buffer containing 300 mM NaCl and 50 mM imidazole, the enzyme was eluted with 20 mM buffer containing 300 mM NaCl and 500 mM imidazole. The combined active fractions were dialyzed with 20 mM buffer and used for the large-scale synthesis of (*S*)-acid **1**.

Effects of pH and temperature on enzyme activity and stability. The effects of pH and temperature on enzyme activity of *S*-amidase were assayed as described below. (A) Effects of pH: Enzyme activity was measured in the following 20 mM buffers at 30 °C: acetate buffer (pH 4.0–6.0), KPB (pH 6.0–7.5), Tris-HCl buffer (pH 7.5–9.0), or Gly-NaOH buffer (pH 9.0–10.5). (B) Effects of temperature: Enzyme activity was measured in 20 mM KPB (pH 7.0) at various temperatures (10–60 °C).

The effects of pH and temperature on enzyme stability of *S*-amidase were assayed as described below. (C) Effects of pH: Enzyme activity was measured in 20 mM KPB (pH 7.0) at 30 °C after a pre-incubation in the following 20 mM buffers for 30 min at 30 °C: acetate buffer (pH 4.0–6.0), KPB (pH 6.0–7.5), Tris-HCl buffer (pH 7.5–9.0), or Gly-NaOH buffer (pH 9.0–10.5). (D) Effects of temperature: Enzyme activity was measured in 20 mM KPB (pH 7.0) at 30 °C after a pre-incubation in 20 mM KPB (pH 7.0) for 30 min at various temperatures (0–70 °C).

Effects of metal ions and inhibitors. Enzyme activity was measured after a pre-incubation with various compounds at a final concentration of 1 or 10 mM for 10 min at 30 °C.

Substrate specificity. Each enzyme activity toward various aliphatic, aromatic, and amino acid amides was determined by the production of either the corresponding carboxylic acid or hydroxamic acid, or by the formation of NH_3 under standard assay conditions. In the quantitative analysis of relative activities toward 2-hydroxy-2-methylpropanamide, 2-methylpropanamide, 2-hydroxypropanamide, or propanamide, enzyme activity was measured using HPLC with a Cosmosil® C_{18}-MS-II column (solvent: 0.1% H_3PO_4 aq./MeCN = 9/1; flow rate: 0.80 mL/min; temperature: 40 °C) for the conversion and with a Sumichiral OA-5000 column [solvent: 2 mM $CuSO_4$ aq./isopronol = 95/5; flow rate: 1.0 mL/min; temperature: 40 °C; detection: 254 nm; t_R = 7.1 min for (*R*)-acid and 8.6 min for (*S*)-acid] for the enantiomeric excess of lactic acid.

Optimization of reaction conditions for the hydrolysis of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide by purified recombinant S*-amidase with the C-His$_6$-tag.* Factors affecting the preparation of acid **1** using the purified recombinant *S*-amidase with the C-His$_6$-tag were examined. Details of the reaction conditions were specific for each case and described in the Figures and Tables. The standard conditions were as follows. The purified enzyme was added in a total volume of 10 mL to a solution of (±)-amide **2** in 500 mM Tris-HCl buffer. A 400-μL aliquot was withdrawn after an incubation for an appropriate period at 30 °C with shaking at 150 rpm, and 200 μL of 0.1 N HCl was added. After centrifugation (20,000 × *g*, 10 min, 4 °C), a 400-μL aliquot of the supernatant was withdrawn. After further centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatant was analyzed by HPLC with a Cosmosil®

C_{18}-MS-II column and Sumichiral OA-5000 column, respectively, as described above.

Kinetic resolution of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide by purified recombinant S-*amidase with the C-His$_6$-tag.* To a solution of (±)-amide **2** (7.85 g, 50.0 mmol) in 500 mM Tris-HCl buffer (pH 8.5), 100 U of purified recombinant *S*-amidase with the C-His_6-tag was added in a total volume of 100 mL. A 400-μL aliquot was withdrawn after an incubation for an appropriate period at 30 °C with shaking at 150 rpm, and 200 μL of 0.1 N HCl was added. After centrifugation (20,000 × *g*, 10 min, 4 °C), a 400-μL aliquot of the supernatant was withdrawn. After further centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatant was analyzed by HPLC with a Cosmosil® C_{18}-MS-II column as described above. After an incubation for 12 h at 30 °C with shaking at 150 rpm, the reaction mixture was extracted with EtOAc five times. The combined organic extract was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was the recovered (*R*)-amide **2** (3.93 g, 50.0%). The residual aqueous layer was acidified to pH 4 by the addition of 2 N HCl and extracted with Et_2O five times. The combined organic extract was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was the formed (*S*)-acid **1** (3.63 g, 45.9%). Based on a HPLC analysis with a Sumichiral OA-5000 column as described above, the enantiomeric excess of (*S*)-acid **1** was determined to be 98.2%, and this could be further enhanced to >99.9% by the recrystallization from hexane/EtOAc for the preparation of an analytical sample of (*S*)-acid **1**. In order to determine the enantiomeric excess of recovered (*R*)-amide **2**, a small portion of (*R*)-amide **2** was hydrolyzed to (*R*)-acid **1** in conc. H_2SO_4 at reflux. Based on a HPLC analysis with a Sumichiral OA-5000 column as described above, the enantiomeric excess of (*R*)-acid **1** was determined to be 99.2%. The enantiomeric excess of (*R*)-amide **2** could be further enhanced to >99.9% by the recrystallization from hexane/EtOAc for the preparation of an analytical sample of (*R*)-amide **2**.

Results

Purification of S-*amidase from* Arthrobacter *sp. S-2*

To purify *S*-amidase from *Arthrobacter* sp. S-2, the culture conditions suitable to induce potent *S*-enantioselective amide **2** hydrolysis activity were examined. As a result, *S*-amidase was constitutively expressed in cells, and glycerol and $(NH_4)H_2PO_4$ were chosen as the sources of carbon and nitrogen, respectively.

After the cultivation of *Arthrobacter* sp. S-2 cells under optimized conditions, *S*-amidase was purified using various column chromatographies as shown in Table 1. The active fraction after Superdex 200 HR 10/30 provided a single band stained on Native-PAGE using the method for the determination of hydroxamic acid formation from acid **1** produced by the hydrolysis of the corresponding amide **2**,[11] which also showed a single band with a molecular mass of 52 kDa on SDS-PAGE. The N-terminal amino acid sequence of the purified enzyme was determined to be Met-Gln-Gly-Leu-Pro-Thr-Arg-Ser-Ser-Asn-Gly-Ala-Ile.

Cloning of the gene coding S-*amidase from* Arthrobacter *sp. S-2*

Based on the information from the N-terminal amino acid sequence, a partial gene coding *S*-amidase was amplified using the LA PCR™ *in vitro* cloning kit. Inverse PCR was carried out to obtain the complete sequence of the gene coding *S*-amidase. The gene coding *S*-amidase consisted of 1389 bp with a high GC content of 63%, and coded for a protein of 462 amino acids (molecular mass of 48,226 Da).

The amino acid sequence of *S*-amidase was compared to those of known enzymes using a BLAST search in the NCBI database, and their alignment was shown in Fig. 2. It showed that the deduced primary structure of *S*-amidase shared approximately 45% identity with several proteins, such as a hypothetical protein from *Actinomycetospora chiangmaiensis* (46.6% identical over 380 amino acids; accession number WP_018332650), a hypothetical protein from *Streptomyces* sp. NRRL-NF-6131 (46.6% identical over 393 amino acids; accession number WP_030303371), a hypothetical protein AJAP_08420 from *Amycolatopsis japonica* (46.4% identical over 394 amino acids; accession number AIG74587), and amidase from *Amycolatopsis orientalis* (45.8% identity over 404 amino acids; accession number WP_016336500). These five proteins displayed highly significant homology, especially between residues 68 and 216 of the *S*-amidase sequence.

Production and purification of recombinant S-*amidase in* E. coli *BL21-CodonPlus®(DE3)-RIPL transformants*

Six different expression systems were constructed and investigated for the expression of the gene coding *S*-amidase in *E. coli*. Each of the *E. coli* BL21(DE3) and *E. coli* BL21-CodonPlus®(DE3)-RIPL strains harboring pEAAS23, pEAAS23-CH, or pEAAS15 were

Table 1. Purification of *S*-amidase from *Arthrobacter* sp. S-2.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	25,900	840	0.0324	100	1
Q Sepharose	1,190	433	0.364	51.5	11.2
Butyl-Toyopearl®	146	232	1.59	27.6	49.0
Gigapite	61.6	192	3.12	22.9	96.1
DEAE-Toyopearl®	3.43	53.2	15.5	6.33	478
Resource PHE	0.363	25.1	69.1	2.99	2130
Superdex 200 HR 10/30	–	0.200	–	0.0237	–

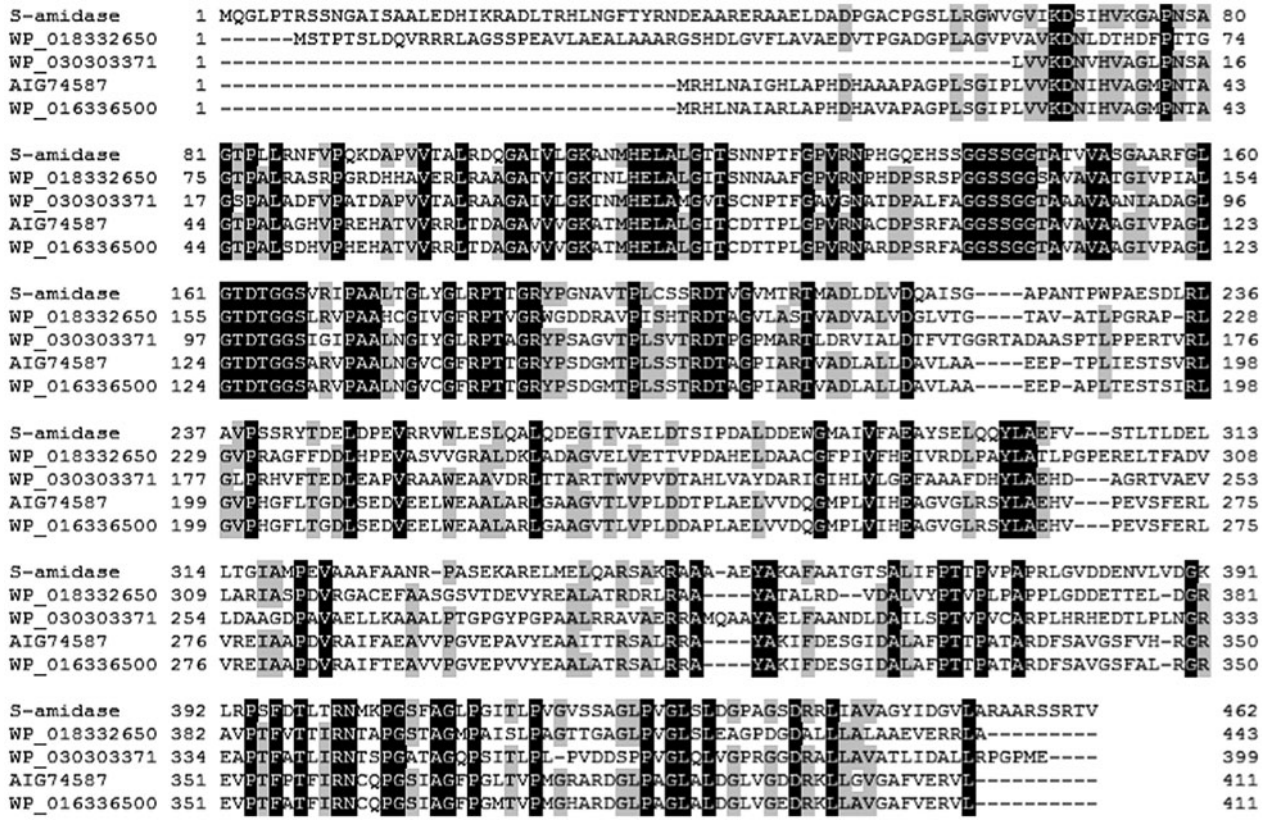


Fig. 2. Alignment of the amino acid sequences of *S*-amidase and other homologous proteins. Identical and conserved amino acids among the sequences are marked in black and gray, respectively. *S*-amidase: *S*-amidase from *Arthrobacter* sp. S-2; WP_018332650: hypothetical protein from *Actinomycetospora chiangmaiensis*; WP_030303371: hypothetical protein from *Streptomyces* sp. NRRL-NF-6131; AIG74587: hypothetical protein AJAP_08420 from *Amycolatopsis japonica*; WP_016336500: amidase from *Amycolatopsis orientalis*.

Table 2. Purification of recombinant *S*-amidase from *E. coli* transformants.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	658	3020	4.59	100	1
Q Sepharose	52.3	1750	33.5	57.9	7.29
Butyl-Toyopearl®	8.87	824	92.9	27.3	20.2
Mono Q® HR 5/5	1.27	275	217	9.11	47.2
Superdex 200 HR 10/30	0.796	177	222	5.86	48.4

grown in LB medium supplemented with appropriate antibiotics using IPTG as an inducer. Of these, *E. coli* BL21-CodonPlus®(DE3)-RIPL transformants harboring pEAAS23 or pEAAS23-CH produced the recombinant enzyme as a soluble fraction, respectively, whereas other *E. coli* transformants did not. These results suggested that codon usage affected the expression level of the gene coding *S*-amidase. Each of the enzyme activities of the *E. coli* BL21-CodonPlus®(DE3)-RIPL transformants harboring pEAAS23 and pEAAS23-CH reached higher than 1510 and 1340 U/L of the culture, which was more than 90- and 80-fold greater than that of *Arthrobacter* sp. S-2 (16.8 U/L), respectively.

In order to characterize the enzyme, recombinant *S*-amidase was purified from *E. coli* BL21-CodonPlus® (DE3)-RIPL harboring pEAAS23 with a recovery of 5.86% by Q Sepharose, Butyl-Toyopearl®, Mono Q HR 5/5, and Superdex 200 HR 10/30 column chromatographies, as shown in Table 2. The active fractions

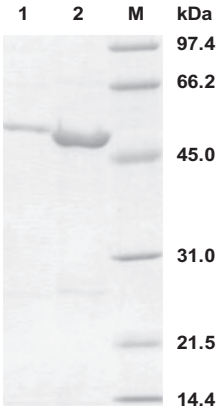


Fig. 3. SDS-PAGE of purified recombinant *S*-amidase. Lane 1: purified recombinant *S*-amidase from *E. coli* transformants; Lane 2: purified recombinant *S*-amidase with the C-His_6-tag from *E. coli* transformants; M: molecular mass standards (phosphorylase b: 97.4 kDa; bovine serum albumin: 66.2 kDa; ovalbumin: 45.0 kDa; carbonic anhydrase: 31.0 kDa; trypsin inhibitor: 21.5 kDa; lysozyme: 14.4 kDa).

Table 3. Purification of recombinant *S*-amidase with the C-His_6-tag from *E. coli* transformants.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	2450	6690	2.73	100	1
Ni Sepharose	24.0	3850	160	57.5	58.7

after Superdex 200 HR 10/30 provided a single band on SDS-PAGE with a molecular mass of 52 kDa as shown in Fig. 3. The molecular mass of the native enzyme was approximately 47 kDa according to gel-filtration chromatography, indicating that the enzyme was a monomer. The purified enzyme catalyzed the hydrolysis of amide **2** to acid **1** at 222 U/mg under standard conditions.

Recombinant *S*-amidase with the C-His_6-tag was purified from *E. coli* BL21-CodonPlus®(DE3)-RIPL harboring pEAAS23-CH with a recovery of 57.5% by nickel affinity chromatography, as shown in Table 3, and its specific activity was 160 U/mg.

Effects of pH and temperature on enzyme activity and stability

In order to characterize *S*-amidase, the effects of pH and temperature on enzyme activity and stability were examined as shown in Fig. 4. The optimum pH for the activity of this enzyme was measured in the buffers described above. The enzyme showed maximum activity at pH 8.5. The reaction was carried out at various temperatures in 20 mM KPB (pH 7.0), and maximum activity was exhibited at 40 °C. After the enzyme had been pre-incubated for 30 min, the remaining activity was assayed. The enzyme was stable at pH values between 8.0 and 10.0 and was also stable up to 30 °C. The enzyme showed 27% residual activity after the incubation at 40 °C and was almost inactivated after the incubation at 60 °C.

Effects of metal ions and inhibitors

Various compounds were investigated for their inhibitory effects on enzyme activity. Enzyme activity was measured under standard conditions after a pre-incubation with various compounds at a final concentration of 1 or 10 mM for 10 min at 30 °C. Enzyme activity was unaffected by the addition of 1 mM of $MgCl_2$, $MnCl_2$, $ZnCl_2$, or $PbCl_2$, or the addition of 10 mM of EDTA. These results indicated that this enzyme had no requirement for metal ions. The enzyme was inhibited by the addition of serine-modifying agents such as phenylmethanesulfonyl fluoride. This result suggested that the serine residue was involved in the active site of this enzyme. This enzyme was also inhibited by the addition of $HgCl_2$ (91.4%), $AgNO_3$ (78.6%), H_3BO_3 (71.1%), $CuCl_2$ (43.0%), or $CuSO_4$ (41.5%). Other inorganic compounds such as LiBr, NaCl, $MgSO_4$, $MgCl_2$, $AlCl_3$, KCl, KI, $CaCl_2$, $CrCl_3$, $MnSO_4$, $MnCl_2$, $FeSO_4$, $FeCl_3$, $Fe(NH_4)_2(SO_4)_2$, RbCl, Na_2MoO_4, $SnCl_2$, and $BaCl_2$ did not influence enzyme activity. Carbonyl reagents such as DL-penicillamine were not inhibitory toward this enzyme.

Substrate specificity

To investigate substrate specificity, purified recombinant *S*-amidase was used to hydrolyze various amides, and its activity was assayed under standard conditions as shown in Table 4. Besides amide **2**, the enzyme was very active toward 2-hydroxy-2-methylpropanamide. However, 2-methylpropanamide, 2-hydroxypropanamide, and

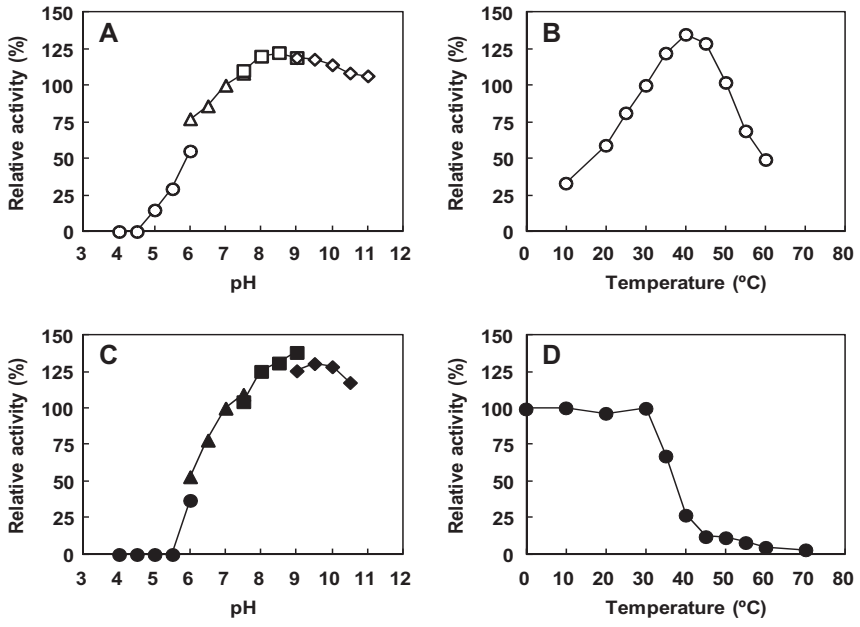


Fig. 4. Effects of pH (A) and temperature (B) on enzyme activity, and of pH (C) and temperature (D) on enzyme stability. The enzyme activity of *S*-amidase was measured in the following 20 mM buffers at 30 °C (A), and in 20 mM KPB (pH 7.0) at various temperatures (10–60 °C) (B). The enzyme activity of *S*-amidase was measured in 20 mM KPB (pH 7.0) at 30 °C after a pre-incubation for 30 min in the following 20 mM buffers at 30 °C (C), and after a pre-incubation for 30 min in 20 mM KPB (pH 7.0) at various temperatures (0–70 °C) (D). The buffers were acetate buffer (○●), KPB (△▲), Tris-HCl buffer (□■), or Gly-NaOH buffer (◇◆) in (A) and (C).

Table 4. Substrate specificity of *S*-amidase from *Arthrobacter* sp. S-2.

Substrate	Relative activity (%)
3,3,3-Trifluoro-2-hydroxy-2-methylpropanamide	100
2-Hydroxy-2-methylpropanamide	59.9
2-Methylpropanamide	9.09
2-Hydroxypropanamide	7.22
Propanamide	0.679

propanamide were hydrolyzed at markedly lower rates than amide **2**. The following compounds were not a substrate for *S*-amidase: 3,3,3-trifluoro-2-hydroxypropanamide, trimethylacetamide, acetamide, butyramide, pentanamide, hexanamide, benzamide, caprolactam, glycine amide, DL-alanine amide, DL-lysine amide, DL-phenylalanine amide, and DL-proline amide. The E[13] values for amide **2** and 2-hydroxy-2-methylpropanamide were 170 and 1.23, respectively.

Optimization of reaction conditions for the hydrolysis of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide by purified recombinant S*-amidase with the C-His$_6$-tag*

In order to optimize reaction conditions, various reaction conditions for the hydrolysis of amide **2** were studied with purified recombinant *S*-amidase with the C-His$_6$-tag, as shown in Table 5. Glycerol and/or 2-mercaptoethanol was added to a reaction mixture (10 mL) containing 5 mmol of amide **2** and 10 U of the purified enzyme at pH 8.5 to stabilize the enzyme, and the reaction was performed for 8 h at 30 °C with shaking (entry 1–4). Glycerol or 2-mercaptoethanol effectively stabilized the enzyme, while the addition of both of them slightly inhibited the reaction. We selected the addition of 2-mercaptoethanol which can be removed more easily by extraction than glycerol, because the additive has to be separated after the reaction. The reaction was carried out at various pHs (entry 4–6). The reaction rates were nearly the same at approximately pH 8.5, the optimum pH for enzyme activity. Therefore, the most effective pH for the reaction was 8.5. The substrate concentration varied, and the yield of the formed acid **1** was measured as shown in Fig. 5. Although the high enantioselectivity of this reaction was maintained up to 2000 mM, the initial reaction rate was gradually decreased by increasing the substrate concentration, and this may have been due to substrate or product inhibition (entry 4, 7, and 8). Although the amount of the enzyme was increased, the reaction could not proceed smoothly (entry 9).

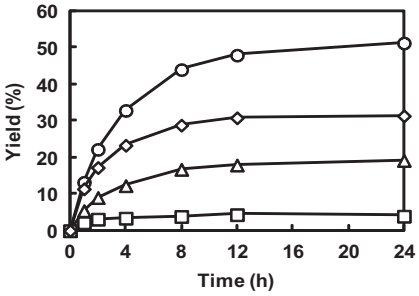


Fig. 5. Enantioselective hydrolysis of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide **2** by purified recombinant *S*-amidase with the C-His$_6$-tag from *E. coli* transformants. The standard conditions were as follows. The purified enzyme was added in a total volume of 10 mL to a solution of (±)-amide **2** in 500 mM Tris-HCl buffer. The reaction was analyzed as described in the Materials and methods after an incubation for an appropriate period at 30 °C with shaking at 150 rpm. ○: 5.00 mmol of (±)-amide **2** and 10.0 U of the enzyme; △: 10.0 mmol of (±)-amide **2** and 10.0 U of the enzyme; □: 20.0 mmol of (±)-amide **2** and 10.0 U of the enzyme; ◇: 10.0 mmol of (±)-amide **2** and 20.0 U of the enzyme.

Kinetic resolution of 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide by purified recombinant S*-amidase with the C-His$_6$-tag*

We then attempted to demonstrate the applicability of the biocatalytic enantioselective hydrolysis of amide **2** to acid **1** as catalyzed by purified recombinant *S*-amidase with the C-His$_6$-tag. The reaction mixture in 100 mL of 500 mM Tris-HCl buffer (pH 8.5) consisted of 50.0 mmol of (±)-amide **2** and 100 U of the purified enzyme. This was incubated at 30 °C with gentle shaking. The progression of the reaction was monitored by a HPLC analysis. The hydrolysis of amide **2** had proceeded by approximately 50% after 12 h. After centrifugation, the supernatant was extracted with ethyl acetate. Only recovered (*R*)-amide **2** could be extracted in this step, and the (*S*)-acid **1** produced remained in the aqueous layer. (*R*)-Amide **2** and (*S*)-acid **1** could be separated easily using only an extraction step. The yield and enantiomeric excess of (*R*)-amide **2** were 50.0 and 99.2%, respectively. (*S*)-Acid **1** could be extracted from the aqueous layer with diethyl ether at

Table 5. Optimization of the reaction conditions of *S*-amidase from *Arthrobacter* sp. S-2.

Entry	Additive		pH	Sub. conc. (mM)	Enzyme activity (U)	Yield (%)
	Glycerol (5%)	2-Mercaptoethanol (5 mM)				
1	−	−	8.5	500	10.0	15.4
2	+	+	8.5	500	10.0	32.3
3	+	−	8.5	500	10.0	44.4
4	−	+	8.5	500	10.0	45.5
5	−	+	8.0	500	10.0	45.0
6	−	+	9.0	500	10.0	43.0
7	−	+	8.5	1000	10.0	16.8
8	−	+	8.5	2000	10.0	3.86
9	−	+	8.5	1000	20.0	28.9

pH 4, and its yield and enantiomeric excess were 45.9 and 98.2%, respectively. The analytical data of (*S*)-acid **1** were as follows. Mp 96.0–98.0 °C; $[\alpha]^{24}_{D} = -20.0$ (*c* 1.00, MeOH); IR (ATR) ν_{max} 3419, 2969, 1736, 1459, 1396, 1328, 1287, 1256, 1188, 1158, 1090, 962, 893, 835, 766, 754, 675 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ: 1.56 (3H, s); ^{13}C NMR (100 MHz, CD_3OD) δ: 18.8, 74.8 (q, $J = 29.2$ Hz), 124.3 (q, $J = 285.0$ Hz), 170.8; HRMS (ESI-TOF) *m*/*z*: calcd. for $C_4H_4F_3O_3^-$ $[M–H]^-$, 157.0118; found, 157.0117. The analytical data of (*R*)-amide **2** were as follows. Mp 110.0–112.0 °C; $[\alpha]^{24}_{D} = -10.0$ (*c* 1.00, MeOH); IR (ATR) ν_{max} 3465, 3276, 1670, 1582, 1455, 1427, 1380, 1280, 1158, 1090, 951, 882, 783, 748 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ: 1.56 (3H, s); ^{13}C NMR (100 MHz, CD_3OD) δ: 18.9, 74.4 (q, $J = 28.2$ Hz), 124.6 (q, $J = 285.2$ Hz), 172.7; HRMS (ESI-TOF) *m*/*z*: calcd. for $C_4H_7F_3NO_2^+$ $[M+H]^+$, 158.0423; found, 158.0437.

Discussion

The preparation of both enantiomers of acid **1** via the two-step whole-cell reaction was previously investigated using *Shinella* sp. R-6 and *Arthrobacter* sp. S-2.[7] *Shinella* sp. R-6 inducibly expressed *R*-enantioselective hydrolysis activity toward amide **2**. In contrast, *Arthrobacter* sp. S-2 constitutively showed hydrolysis activity toward amide **2** and hydrolyzed it *S*-enantioselectively. Although *R*-enantioselective amidase acting on amide **2** had already been purified from *K. oxytoca* PRS1,[5,6] no enzymes exhibiting *S*-enantioselective hydrolysis activity toward amide **2** have been reported to date. Therefore, we herein purified the *S*-enantioselective amidase acting on amide **2** from *Arthrobacter* sp. S-2, and cloned the gene coding this enzyme. The purified recombinant *S*-amidase from *E. coli* transformants was shown to be applicable to the kinetic resolution of (±)-amide **2** to produce (*S*)-acid **1** with high enantiomeric excess.

The amino acid sequences of five proteins including *S*-amidase from *Arthrobacter* sp. S-2, as shown in Fig. 2, shared the conserved regions between residues 68 and 216 of the *S*-amidase sequence. These correlated with the amidase conserved motifs, characterized as the amidase signature (AS) family.[14,15] This family shows a highly significant level of homology, especially approximately 130 amino acids known as the AS sequence (Pfam family: PF01425).[4] Furthermore, comparisons with related proteins, X-ray structures of which were available, including amidase from *Thermus thermophilus* HB8 (34.7% identity over 444 amino acids; accession number 2DC0_A), peptide amidase from *Stenotrophomonas maltophilia* (31.8% identity over 431 amino acids; accession number 1M22_A),[16] amidase from *Rhodococcus* sp. N-771 (29.9% identity over 412 amino acids; accession number 3A1K_A),[17] and malonamidase E2 from *Bradyrhizobium japonicum* (26.7% identity over 454 amino acids; accession number 1OCK_A)[18] indicated that Ser-*cis*Ser-Lys, the highly conserved catalytic triad of the AS family, was also found in *S*-amidase (Lys68, Ser143, Ser167), as shown in Fig. 6. Thus, we suggested that *S*-amidase from *Arthrobacter* sp. S-2 was a member of the AS family.

An assessment of the substrate specificity of *S*-amidase from *Arthrobacter* sp. S-2 revealed that the substitution of the trifluoromethyl group in amide **2** with a methyl group resulted in a 0.60-fold reduction in activity. Furthermore, truncation of the trifluoromethyl and/or hydroxy group in amide **2** decreased activity, whereas truncation of the methyl group resulted in no activity. More simple amides such as aliphatic and aromatic amides, which can be efficiently hydrolyzed by the other microbial amidases, have not been hydrolyzed. These results indicated that the enzyme was specific to amide **2** and showed unique substrate specificity.

The properties of *S*-amidase from *Arthrobacter* sp. S-2 were shown to differ from those of *R*-amidase from

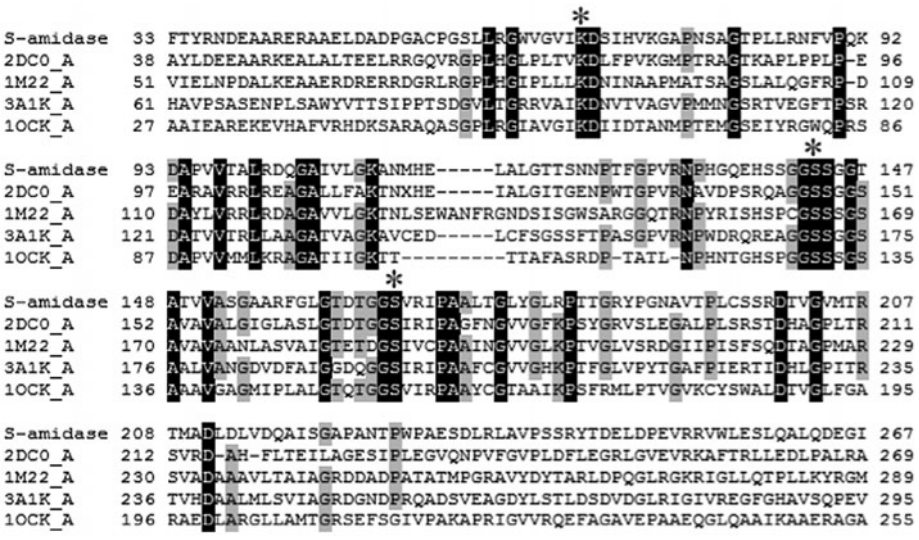


Fig. 6. Partial alignment of amino acid sequences of *S*-amidase and other related proteins at regions known to be the most highly conserved among the AS family. *S*-amidase: *S*-amidase from *Arthrobacter* sp. S-2; 2DC0_A: amidase from *Thermus thermophilus* HB8; 1M22_A: peptide amidase from *Stenotrophomonas maltophilia*; 3A1K_A: amidase from *Rhodococcus* sp. N-771; 1OCK_A: malonamidase E2 from *Bradyrhizobium japonicum*. Three residues that constitute the putative catalytic triad are marked by asterisks.

K. oxytoca PRS1.[5,6] For example, *S*-amidase exhibited its maximum activity at 40 °C, and was almost inactivated after being incubated at 60 °C, whereas *R*-amidase was reported to be heat-stable with maximum activity at 70 °C. On the other hand, the specific activity of *S*-amidase toward amide **2** (222 U/mg) was markedly higher than that of *R*-amidase (2.7 U/mg). Although the specific activity of *S*-amidase with the C-His_6-tag (160 U/mg) was slightly lower than that of *S*-amidase, the high activities of *S*-amidase with/without the C-His_6-tag may enable the efficient production of (*S*)-acid **1** at high substrate concentrations.

Author contributions

K. Fuhshuku, T. Nishii, A. Ishii, and Y. Asano conceived the project. K. Fuhshuku, S. Watanabe, and Y. Asano designed and performed research. K. Fuhshuku, S. Watanabe, and Y. Asano wrote the manuscript. All authors reviewed and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was partly supported by a Grant-in-Aid for Scientific Research (A) (23248015) from Japan Society for the Promotion of Science (JSPS) to Y. Asano.

References

[1] Nie J, Guo H-C, Cahard D, Ma J-A. Asymmetric construction of stereogenic carbon centers featuring a trifluoromethyl group from prochiral trifluoromethylated substrates. Chem. Rev. 2011;111:455–529.

[2] Parker JS, Bower JF, Murray PM, Patel B, Talavera P. Kepner-Tregoe decision analysis as a tool to aid route selection. Part 3. Application to a back-up series of compounds in the PDK project. Org. Process Res. Dev. 2008;12:1060–1077.

[3] Menzel K, Machrouhi F, Bodenstein M, Alorati A, Cowden C, Gibson AW, Bishop B, Ikemoto N, Nelson TD, Kress MH, Frantz DE. Process development of a potent bradykinin 1 antagonist. Org. Process Res. Dev. 2009;13:519–524.

[4] Sonke T, Kaptein B. Hydrolysis of amides. In: Drauz K, Gröger H, May O, editors. Enzyme catalysis in organic synthesis. 3rd ed. Vol. 2. Weinheim: Wiley-VCH; 2011. p. 561–650.

[5] Shaw NM, Naughton A, Robins K, Tinschert A, Schmid E, Hischier M-L, Venetz V, Werlen J, Zimmermann T, Brieden W, de Riedmatten P, Roduit J-P, Zimmermann B, Neumüller R. Selection, purification, characterisation, and cloning of a novel heat-stable stereo-specific amidase from *Klebsiella oxytoca*, and its application in the synthesis of enantiomerically pure (*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acids and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide. Org. Process Res. Dev. 2002;6:497–504.

[6] Shaw NM, Naughton AB. The substrate specificity of the heat-stable stereospecific amidase from *Klebsiella oxytoca*. Tetrahedron. 2004;60:747–752.

[7] Fuhshuku K, Watanabe S, Nishii T, Ishii A, Asano Y. Efficient preparation of both enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid catalyzed by *Shinella* sp. R-6 and *Arthrobacter* sp. S-2. J. Mol. Catal. B: Enzym. 2014;102:115–119.

[8] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72:248–254.

[9] Davis BJ. Disc electrophoresis. II. Method and application to human serum proteins. Ann. NY Acad. Sci. 1964;121:404–427.

[10] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–685.

[11] Fournand D, Pirat J-L, Bigey F, Arnaud A, Galzy P. Spectrophotometric assay of aliphatic monohydroxamic acids and α-, β-, and γ-aminohydroxamic acids in aqueous medium. Anal. Chim. Acta. 1997;353:359–366.

[12] Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izawa Y, Nakamura K, Harashima K. Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. J. Bacteriol. 1990;172:6704–6712.

[13] Chen CS, Fujimoto Y, Girdaukas G, Sih CJ. Quantitative analyses of biochemical kinetic resolutions of enantiomers. J. Am. Chem. Soc. 1982;104:7294–7299.

[14] Chebrou H, Bigey F, Arnaud A, Galzy P. Study of the amidase signature group. Biochim. Biophys. Acta. 1996;1298:285–293.

[15] Mayaux J-F, Cerbelaud E, Soubrier F, Yeh P, Blanche F, Pétré D. Purification, cloning, and primary structure of a new enantiomer-selective amidase from a *Rhodococcus* strain: Structural evidence for a conserved genetic coupling with nitrile hydratase. J. Bacteriol. 1991;173:6694–6704.

[16] Neumann S, Kula MR. Gene cloning, overexpression and biochemical characterization of the peptide amidase from *Stenotrophomonas maltophilia*. Appl. Microbiol. Biotechnol. 2002;58:772–780.

[17] Ohtaki A, Murata K, Sato Y, Noguchi K, Miyatake H, Dohmae N, Yamada K, Yohda M, Odaka M. Structure and characterization of amidase from *Rhodococcus* sp. N-771: insight into the molecular mechanism of substrate recognition. Biochim. Biophys. Acta. 2010;1804:184–192.

[18] Shin S, Lee T-H, Ha N-C, Koo HM, Kim S-Y, Lee H-S, Kim YS, Oh B-H. Structure of malonamidase E2 reveals a novel Ser-*cis*Ser-Lys catalytic triad in a new serine hydrolase fold that is prevalent in nature. EMBO J. 2002;21:2509–2516.