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An Enantioselective Amidase from *Burkholderia multivorans* for the Stereoselective Synthesis of Esfenvalerate

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Using racemic (R,S)-2-(4-chlorophenyl)-3-methylbutyramide, an intermediate for the chiral pyrethroid insecticide Esfenvalerate, as a sole nitrogen source in a minimal medium, several strains with high enatioselectivity (≥98%) were isolated by enrichment techniques. One of the strains, LG 31-3, was identified as *Burkholderia multivorans*, based on physiological and morphological tests by a standardized Biolog station for carbon source utilization. A novel amidase was purified from *B. mutivorans* LG 31-3 and characterized. The enzyme exhibited (S)-selective amidase activity on racemic (R,S)-2-(4-chlorophenyl)-3-methylbutyramide. Addition of the racemic amide induced the production of the enantioselective amidase. The molecular mass of the amidase on SDS-PAGE analysis was shown to be 50 kDa. The purified amidase was subjected to proteolytic digestion with a modified trypsin. The N-terminal and internal amino acid sequences of the purified amidase showed a high sequence homology with those deduced from a gene named YP_366732.1 encoding indole acetimide hydrolase from *Burkholderia* sp. 383.

Keywords: Nitrile-hydrolyzing enzyme, amidase, pyrethroid insecticides, *Burkholderia* sp., stereoselectivity

Introduction

Hydrolases such as lipases, esterases, and amidases are an important class of biocatalysts, which are frequently used in organic synthesis of chiral compounds [4, 5]. In particular, amidases (acylamide aminohydrolase, E.C. 3.5.1.4) have received continued attention in the preparation of optically active amino acids, natural and unnatural, and 2-arylpropionic acids [11, 21]. They catalyze the hydrolysis of carboxylic amides to generate carboxylic acids and ammonia. Some enantioselective amidases are known to be involved in the nitrile metabolism of microorganisms [15, 22].

Esfenvalerate [(S)-α-cyano-3-phenoxybenzyl(S)-2-(-4-chlorophenyl)-3-methylbutyrate] is one of the pyrethroid insecticides (Fig. 1). The acid moiety, (S)-2-(4-chlorophenyl)-3-methylbutyric acid, (S)-**2**, could be obtained by enantioselective hydrolysis of various starting materials such as esters, amides, and nitriles (Fig. 2). Previously, two reports dealt with the preparation of (S)-**2** by enantioselective hydrolysis of (R,S)-2-(4-chlorophenyl)-3-methylbutyronitrile by *Pseudomonas* sp. B21C9 [16] and *Pseudomonas putida* NRRL-18668 [7]. However, the reaction was catalyzed by the whole cells and the active amidase enzymes were not purified.

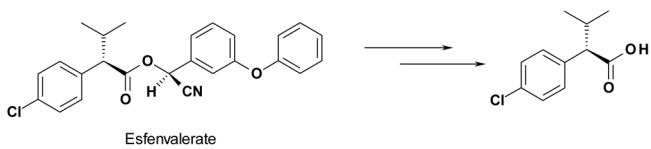

Fig. 1. Structure of Esfenvalerate and its intermediate, (S)-2-(4-chlorophenyl)-3-methylbutyric acid.

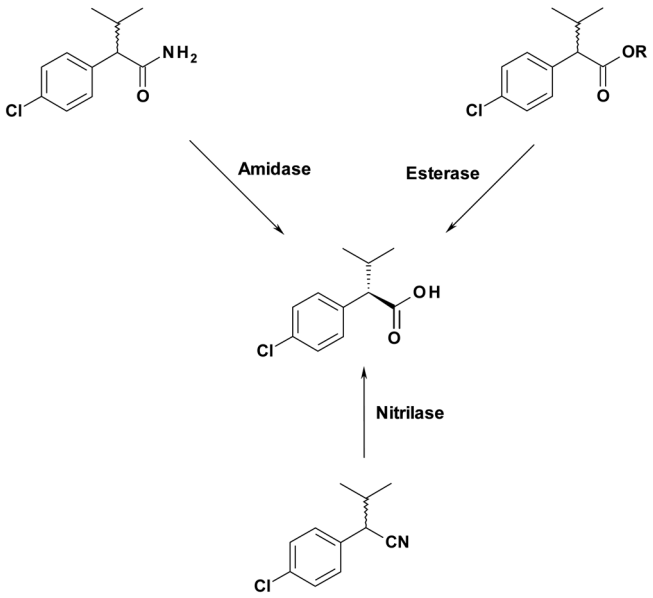

Fig. 2. Various enzymatic hydrolytic kinetic resolution methods for the synthesis of Esfenvalerate intermediate, (S)-2-(4-chlorophenyl)-3-methylbutyric acid.

Traditionally, various techniques such as enrichment cultivation and staining methods were employed to isolate useful amidases from amide- and/or nitrile-degrading microorganisms [8, 14, 24]. However, the discovery of stereoselective biocatalysts from nature as well as an established gene library still remains a challenging task for researchers. Although various amidases showed a wide range of substrate specificities, there are few reports on the hydrolysis of amides with a bulky substituent like (R, S)-2-(4-chlorophenyl)-3-methylbutyramide, (R, S)-**1**.

In this work, strain *B. multivorans* LG 31-3, with an (S)-stereoselective amidase enzyme activity, was isolated from the environment by the enrichment technique, with racemic (R,S)-**1** as a sole nitrogen source. An enantioselective amidase from *B. multivorans* LG 31-3 was purified and applied to the stereoselective hydrolysis of racemic (R, S)-**1**.

Materials and Methods

All chemicals were purchased from Aldrich and Sigma (St. Louis, MO, USA) at the highest purity available. Racemic (R, S)-**1** (fenvaleramide, FAA), (R)-**2**, (S)-**2**, racemic methyl **(3)**, and ethyl **(4)** esters were provided by LG Chemicals (Daejon, Korea). Trypsin modified for protein sequencing from bovine pancreases was purchased from Roche (Penzberg, Germany). Commercial esterases and lipases for hydrolysis of racemic esters (R, S)-**3** and **4** were from the Roche Chiral Screening Kit (Penzberg, Germany).

Microbial Screening for Amidase Activity

In order to isolate microorganisms producing an amidase that has a stereoselective substrate specificity and can be used for an optical resolution of a racemic mixture, apple farm soils (Kimcheon, Korea), which were exposed to fenvalerate for several years, as well as water samples from wastewater treatment facilities were used. The soil and water samples were serially diluted with a sterile solution (0.1 M potassium phosphate buffer, pH 7.2) and inoculated in a liquid minimal medium (SFAA medium) containing racemic FAA as a sole nitrogen source at 35°C and 200 rpm. SFAA medium is composed of the following ingredients; FAA 2 g, glucose 2 g, KH_2PO_4 3 g, K_2HPO_4 7 g, $MgSO_4$-$7H_2O$ 0.1 g, sodium citrate 0.5 g, vitamin solution 10 ml per liter, and trace metal solution 5 ml per liter (pH 7.0). The turbidity of culture broths was observed with the naked eye. Then culture broths that showed the growth of strains were transferred into a new SFAA medium and cultivated and plated on a solid minimal medium containing FAA as a sole nitrogen source (1.5% agar). Colonies grown on the media were judged to decompose FAA, and purely cultured again to select colonies in which the starting FAA had a conversion ratio of 50% with HPLC analysis (if completely enantioselective, 50% conversion is the maximum). Trace metal solution (1 ml per liter, filtered) contained $FeSO_4$-$7H_2O$ 200 mg, $ZnCl_2$ 100 mg, $MnSO_4$-$5H_2O$ 30 mg, $Na_2B_4O_7$-$10H_2O$ 100 mg, $CoCl_2$-$6H_2O$ 20 mg, $CuSO_4$-$5H_2O$ 10 mg, NiCl-$6H_2O$ 10 mg, Na_2MoO_4-$2H_2O$ 10 mg, and $CaCl_2$-$2H_2O$ 10 mg per liter. Vitamin solution (1 ml per liter, filtered) contained Thiamine-HCl 4 mg, riboflavin 2 mg, pantothenic acid 4 mg, pyridoxine-HCl 4 mg, *p*-aminobenzoic acid 4 mg, nicotinic acid 4 mg, inositol 20 mg, and biotin 0.02% solution 100 µl per liter.

Cultivation of *B. multivorans* LG 31-3

B. multivorans LG 31-3 was inoculated in a complex medium and cultured at 35°C and 200 rpm for 24 h (medium D; glucose 4 g, (R, S)-**1** (FAA) 2 g, K_2HPO_4 10 g, NaH_2PO_4 5 g, NaCl 0.5 g, $CaCl_2$-$2H_2O$ 0.02 g, $(NH_4)_2SO_4$ 3.5 g, yeast extract 0.5 g, $MgSO_4$-$7H_2O$ 0.3 g, and trace metal solution 10 ml per liter, pH 7.5). The culture broth was centrifuged and the cell pellet was suspended in 25 ml of a 0.1 M Tris-HCl solution (pH 8.0). Then 3 ml of cell suspension and 30 µl of FAA stock solution (in methanol at a concentration of 105.6 mg/ml) were mixed for a resting cell biotransformation (30°C). The resulting reaction mixtures were analyzed with HPLC.

Purification of (S)-Stereoselective Amidase from *B. multivorans* LG 31-3

Step 1: Preparation of cell-free extracts. Washed cells from 7.5 L of culture broth (in medium D) were suspended in 200 ml of 50 mM potassium phosphate buffer (KPB buffer A, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA) and then disrupted by a BeadBeater (Biospec, OK, USA). The cell debris was removed by centrifugation.

Step 2: Ammonium sulfate precipitation. Ammonium sulfate

was added to the resultant cell extract suspension to give 35%, 55%, and 75% saturation solutions. Each fraction was dissolved in buffer A and dialyzed against KPB buffer A solution (MWCO 12,000 Da). The resultant dialyzed solutions were reacted with 10 µl of FAA stock solution in 1 ml of 50 mM Tris-HCl (pH 7.8). At different time intervals, 100 µl of samples were taken from the reaction mixture and 50 µl of 1 M H_3PO_4 was added to stop the reaction, and 850 µl of acetonitrile was added before HPLC analysis.

Step 3: 35% ammonium saturation precipitate solution was desalted with PD-10 (GE Healthcare, Buckinghamshire, UK) and concentrated using a YM10 membrane (Millipore, Billerica, MA, USA) and applied to ion-exchange chromatography using a Mono Q HR 5/5 column (Amersham Biosciences, Buckinghamshire, UK) equilibrated with 50 mM KPB (1 mM DTT, 1 mM EDTA, pH 7.2). Protein was eluted from the column with 50 ml of 50 mM KPB containing 1 M KCl (pH 7.2).

Step 4: Hydrophobic interaction chromatography (HIC). HIC was performed on phenyl Sepharose HR 5/5, where 2 ml of sample containing 1 M ammonium sulfate was loaded and bound proteins were desorbed by washing with 50 mM KPB (1 mM DTT, 1 mM EDTA, pH 7.2).

All purified proteins were concentrated using a Millipore Amicon Concentrator (Amincon 8200; Billerica, MA, USA). All purification steps were performed at a temperature lower than 5°C. Protein was determined by the bicinchoninic acid assay (BCA assay) using bovine serum albumin as the standard.

N-Terminal Sequencing of a Purified Protein and Peptides After Trypsin Digestion

Peptides were formed by proteolytic digestion of the purified protein in the presence of modified trypsin (sequencing grade; Roche). First, 10 µl of a trypsin solution (concentration 1 µg/µl, 50 mM KPB, 1 mM mercaptoethanol, 1 mM EDTA, pH 8.0) was mixed with 100 µl of the protein solution. The resultant mixture was incubated at 37°C for 4 h. Then 50 µl of the reaction solution was separated by reverse-phase HPLC (1 ml/min, 210 nm, Tosoh Capcell Pak 4.9 × 300 mm; elution condition: solvent A-0.1% Trifluoroacetic acid (TFA) water, solvent B-0.1% TFA acetonitrile, linear gradient 100% solvent B in 60 min). Eluted peptides were fractionated manually (retention time at 31.6 and 37.1 min) and concentrated and subject to N-terminal sequencing (Applied Biosystems, Foster City, CA, USA).

Sequence Alignments of N-Terminal and Internal Sequences

The amino acid sequences obtained were compared with those in the GenBank databases using the BLAST program [1]. The sequences were aligned using multiple sequence alignment software, CLUSTAL X ver. 2.1 [13].

HPLC Analysis for the Determination of *S*- and *R*-Amides

The hydrolysis reaction of (R,S)-**1** was examined with 10 mM (R,S)-**1** using standard conditions. During the purification of the amidase from LG 31-3, the enzyme assay was carried out with (R,S)-**1** as a substrate. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.8), 10 mM (R,S)-**1**, and an aliquot of the enzyme.

Samples were periodically withdrawn from separate reaction mixtures and centrifuged to remove insoluble materials and analyzed on HPLC. (R,S)-**1** and (R,S)-**2** were analyzed for the conversion ratio through reverse-phase HPLC analysis (acetonitrile/water = 7/3 (v/v)) with HPLC (C18 Optimapak; RSTech, Daejon, Korea).

Chiral HPLC Analysis for Enzyme Reactions

The 50 strains showing hydrolytic ability were further analyzed on a chiral HPLC column (Chiral AGP ChromTech, Congleton, UK). Elution conditions for chiral analysis were Na_2HPO_4 aqueous solution (10 mM, pH 6.0)/ethanol = 95/5 (vol%); flow rate 0.9 ml/min; 230 nm; retention times (S)-**2** 8.0 min, (R)-**2** 6.0 min, (S)-**1** 20 min, and (R)-**1** 16 min. Enantioselectivity was calculated using the following equation: (ee_p = {((S)-acid – (R)-acid)/((S)-acid + (R)-acid)} × 100%).

Electrophoresis

The proteins were separated by SDS-PAGE (10% acrylamide) as described by Laemmli [12]. After separation by SDS-PAGE, the proteins were detected by staining the gel with Coomassie blue R250. Native gel (12% acrylamide) was prepared according to standard protocols [20]. For N-terminal sequencing of purified proteins after SDS-PAGE, the protein was blotted on a PVDF membrane (CAPS buffer, pH 11.0).

Results

Screening of Enantioselective Amidase-Producing Microorganisms

It can be envisioned that esterases and lipases are used to obtain (S)-**2** by enantioselective hydrolysis of racemic methyl and ethyl (R, S)-2-(4-chlorophenyl)-3-methylbutyric ester, (R,S)-**3** and **4**. Esterases and lipases commercially available were screened for their ability to hydrolyze the racemic esters (R,S)-**3** and **4**. It was found that no significant conversion was achieved. Next, we aimed to screen microorganisms from the soils producing esterases and lipases that were capable of hydrolyzing esters of (R,S)-**1**. Still, the enantioselective esterase-producing microorganisms were not obtained with racemic (R,S)-**3** as a sole carbon source. Instead, we tried to screen amidase-producing strains. The basic concept of the screening is to use (R,S)-**2** as a nitrogen source in an enrichment cultivation.

About 200 strains formed colonies on a solid SFAA medium and were preserved. Among the isolated strains, including LG 31-3, six strains showed excellent stereoselectivity toward S-enantiomer (with LG 31-3 *S*/*R* selectivity was 99/1

Table 1. Screening of enantioselective amidase-producing strains by HPLC analysis.

Strains	SFAA 9-4	SFAA 12-5	SFAA 31-1	MC12-1	LG 31-3	A118-2
Conversion[a] (%)	41	36	35	39	39	31
S/*R* selectivity (*ee$_p$)[b]	97.9	97.8	97.5	97.9	98.0	96.7

[a]Conversion is based on racemic amide (R,S)-**1**.

[b](*ee$_p$) = enantiomeric excess of the product expressed as in { [(S)-2 – (R)-2] / [(S)-2 + (R)-2] × 100}.

at 39% conversion) (Table 1). LG 31-3 was chosen as the best strain for further study. A fingerprinting analysis with the Biolog system kit (Biolog Inc., Hayward, CA, USA) identified strain LG 31-3 as *Burkholderia multivorans* and the strain was deposited as KCTC 10920BP (Korean Collection of Type Cultures, Daejon, Korea).

Purification of the Enantioselective Amidase from *B. multivorans* LG31-3

An amidase activity against (R,S)-**1** was detected in *B. multivorans* LG31-3. To investigate the stereoselectivity of the hydrolytic activity toward the substrate, the amidase was purified from the cell-free extract of *B. multivorans* LG 31-3 as described in Materials and Methods. Various amides (aliphatic and aromatic) were employed for inducing amidases (data not shown).

However, to induce enantioselective amidases, (R,S)-**1** was used at 0.2% (w/v) concentration besides yeast extract (0.05% (w/v)) as nitrogen sources. It was found that an enantioselective amidase was precipitated in 0–35% saturation ammonium sulfate. Different volume amounts of the fraction of 0–35% saturation were added to the enzyme assay mixture. The analysis of the resultant mixture is shown in Fig. 3. The fact that the reactions were slowed down after the conversion of racemic (R,S)-**1** reached 50% indicates the precipitated enzyme in 0–35% saturation ammonium sulfate could be enantioselective. After two chromatography steps (IEC and HIC), the purity of the enzyme was confirmed by migration of the protein as a single band corresponding to a molecular mass of 50 kDa on SDS-PAGE (Fig. 4).

Proteolytic Digestion of the Amidase and N-Terminal Sequencing

After the native polyacrylamide gel electrophoresis was performed, bands were excised from a native polyacrylamide gel and activity was shown to be enantioselective, and the the corresponding protein was subject to digestion with the modified trypsin. The peptides were separated on reverse-phase HPLC and two fractions were collected manually (31.6 and 37.1 min) (Fig. 5). The peptides were subject to N-

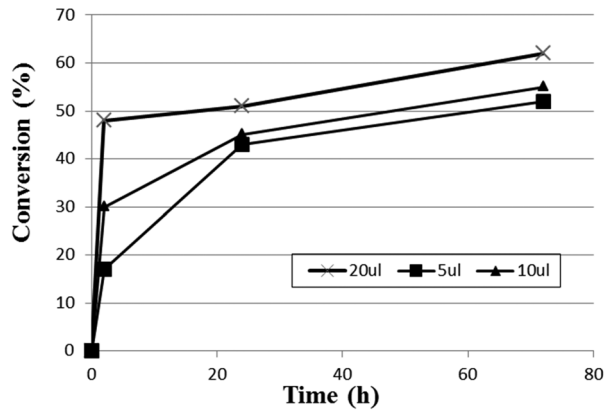

Fig. 3. Time course of enantioselective hydrolysis of racemic (R, S)-2-(4-chlorophenyl)-3-methylbutyramide with an amidase from *B. multivorans* LG 31-3.

The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.8), 10 mM (R,S)-**1**, and an aliquot of the enzyme. Different aliquots of 0–35% ammonium sulfate saturation were added to the standard enzymatic reaction mixture.

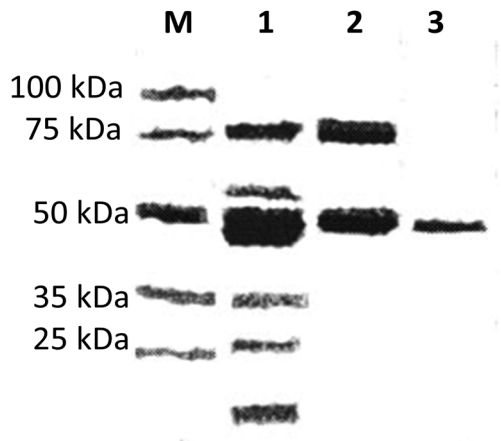

Fig. 4. SDS/PAGE of the purified amidase from *B. multivorans* LG 31-3.

Protein bands were detected by staining with Coomassie brilliant blue. Lane, M: marker proteins; lane 1: ion-exchange chromatography; lane 2: hydrophobic interaction chromatography; lane 3: the purified enzyme.

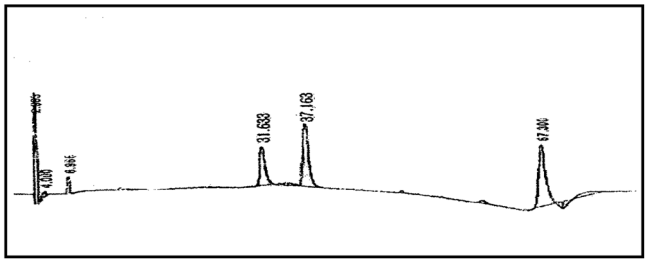

Fig. 5. HPLC chromatogram after proteolytic digestion of the purified amidase from *B. multivorans* LG 31-3 with the modified trypsin.

terminal and internal amino acid sequencing, yielding the following results: N-terminal amino acid sequence, TTLGSLTLTEARHALRREF (31.6 min); Internal amino acid sequence, VTRPVRLALPRTTFWRGLAADVD (37.1 min).

BLAST Search of N-Terminal and Internal Sequences of the Enantioselective Amidase from *B. multivorans* LG 31-3

Two separate BLAST searches with N-terminal and internal sequences of the purified amidase from *B. multivorans* LG 31-3 retrieved two matches. Alignment with the protein databases showed that the N-terminal and internal amino acid sequences of the enantioselective amidase from *B. multivorans* LG 31-3 were similar to those of putative indole acetimide hydrolase from *Burkholderia* sp. 383 (NCBI Reference Sequence: YP_366732.1). Fig. 6 shows the comparison of the N-terminal and internal sequences of the enantioselective amidase from *B. multivorans* LG 31-3 and those of putative

a). 31.6 min. N-terminal sequence : TTLGSLTLTEARHAILRREF

LG 31-3 **TTLGSLTLTEARHA I LRREF**

YP_366732.1 **PTLGTLTLTEARHALLRREF**

b). 37.1 min. Internal sequence : VTRPVRLALPRTTFWRGLAADVD

LG 31-3 **VTRPVRLALPRTTFWRGLAADVD**

YP_366732.1 **TTRPVRLA**

Fig. 6. Comparison of N-terminal and internal amino acid sequences of the enantioselective amidase from *B. multivorans* 31-3.

Shaded areas indicate the difference of amino acids between the two amidases.

indole acetimide hydrolase from *Burkholderia* sp. 383. Both sequences gave the highest scores in the BLAST (N-terminal sequence, 85%: 17 residues out of 20; internal sequence, 83%: 19 residues out of 23). The putative indole acetimide hydrolase consists of 500 amino acids, which corresponds to the molecular mass of 50 kDa of the purified amidase from *B. multivorans* LG 31-3, which was confirmed by SDS-PAGE analysis.

Discussion

Hydrolytic kinetic resolution using hydrolases is still known to be an effective means to obtain chiral compounds for several advantages [5, 18]. As the first attempt, we started the screening of commercially available esterases and lipases with racemic methyl or ethyl (R,S)-2-(4-chlorophenyl)-3-methylbutyrate. We were not able to find an enzyme that was active toward the ester substrates. The bulky isopropyl moiety seemed to hinder enzymatic hydrolysis with esterases and lipases. The bulky nature of the substituent on the α-carbon restricted the effectiveness of esterases and lipases [19].

Techniques for isolating new biocatalysts, such as metagenomics and directed evolution, are recognized as powerful tools to screen and improve industrial biocatalysts [3]. Still, enantioselective amidases from the enrichment techniques are desired. Substrate-directed screening has been reported for screening enantioselective esterases by various research groups, by employing bulky substrate esters such as dibenzoyl tartrate [25] and acetoxy mandelate [10] as the sole carbon source for screening a novel esterase or lipase and excluding known ones. Several reports were published that employed racemic mixtures as nitrogen sources [9, 23]. However, in the examples mentioned above, the screening substrates employed for isolation of the microorganisms were different from the reaction substrates for the target reactions.

In the literature, two reports concerned the enantioselective hydrolysis of racemic (R,S)-2-(4-chlorophenyl)-3-methylbutyronitrile [7, 16]. It appeared that the microbial hydrolysis proceeded *via* stepwise reactions involving a nitrile hydratase with poor (S)-selectivity and an amidase with strict (S)-selectivity. However, the suspected amidases were not purified and their amino acid sequences were not released. In order to isolate an enantioselective amidase, (R,S)-**1** was employed instead of (R,S)-2-(4-chlorophenyl)-3-methylbutyronitrile. In this study, we purified *S*-stereoselective amidase acting on racemic amide, (R,S)-**1**, to produce (S)-**2** with high optical purity.

Partial sequence analysis of the N-terminal and internal amino acid sequences of the purified amidase revealed homology to those of putative indole acetimide hydrolase from *Burkholderia* sp. 383, which catalyzes the conversion of indole-3-acetamide to indole-3-acetic acid, auxin, a phytohormone. Additionally, the observation that the molecular masses of the purified amidase and indole-3-acetamide hydrolase amount to approximately 50 kDa suggests that they can catalyze the similar kind of reactions. In the process of cloning the nitrile hydratase, the amidase was found in the cluster of genes co-transcribed by a single mRNA [17]. An amidase from *P. chlororaphis* B23, which was shown to be enantioselective toward (R,S)-**1** as well as 2-arylpropionic acids, is a homodimer (MW 104 kDa) and its amino acid sequence does not show homology to that of the amidase from *B. multivorans* LG 31-3 [6]. The amidase from *P. chlororaphis* B23 originally was not isolated using a bulky amide as a single nitrogen source, but by using an aliphatic nitrile for screening nitrile hydratases for acrylamide production [2, 17]. Compared with the previous reports, this is the second report on the examples that present the stereoselective amidase useful for the optical resolution of a racemic amide compound with a bulky substituent at the α-carbon.

In conclusion, the amidases that have partial primary sequences similar to those of the enantioselective amidase from *B. multivorans* LG 31-3 have never been reported. To our knowledge, the enantioselective amidase from *B. multivorans* LG 31-3 is the first enantioselective amidase isolated by directly using a racemic amide compound with a bulky substituent at the α-carbon as a sole nitrogen source. It is expected that the enantioselective amidase from strain LG 31-3 will be useful for the optical resolution of other racemic mixtures other than racemic FAA.

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