Enzyme and Microbial Technology 43 (2008) 276–283

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

A novel D-stereoselective amino acid amidase from *Brevibacterium iodinum*: Gene cloning, expression and characterization

Hidenobu Komeda, Yasuhisa Asano*

Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

ARTICLE INFO

Article history:
Received 13 January 2008
Received in revised form 7 March 2008
Accepted 10 March 2008

Keywords:
D-Amino acid
Amidase
D-Phenylalanine
Brevibacterium iodinum
Class A β-lactamase

ABSTRACT

The gene encoding D-stereoselective amino acid amide amidohydrolase (D-amino acid amidase, named $DaaA_{Bi}$) was cloned from a chromosomal DNA library of *Brevibacterium iodinum* TPU 5850 and sequenced. The gene, $daaA_{Bi}$, encoded a protein composed of 266 amino acids with a M_r of 30035. The deduced amino-acid sequence of the $daaA_{Bi}$ gene did not exhibit any similarity with any other previously reported D-amino acid amidases, but did show similarity with hypothetical class A β-lactamases. $DaaA_{Bi}$ protein was produced in *Escherichia coli*, purified to electrophoretic homogeneity, and characterized. The purified enzyme was about 290,000 based on gel filtration chromatography and about 30,000 based on SDS-polyacrylamide gel electrophoresis, suggesting that the enzyme is active as a decamer with identical subunits. $DaaA_{Bi}$ showed maximum activity at pH 7.2 and 35 °C. It exhibited strict D-stereoselective hydrolyzing activity towards a broad range of D-amino acid amides including D-methioninamide, D-lysinamide, D-glutaminamide, and D-phenylalaninamide, while L-amino acid amides, peptides composed of L- or D-amino acids, and β-lactam compounds could not serve as substrates for the enzyme. Almost complete hydrolysis of D-phenylalaninamide with highly strict D-stereoselectivity was achieved in 3 h from 180 mM of DL-phenylalaninamide using the purified $DaaA_{Bi}$ enzyme.

1. Introduction

Enantiomerically pure D-amino acids are versatile chiral building blocks for the synthesis of pharmaceuticals, agrochemicals, and food or feed additives, and several enzymatic methods have therefore been developed to synthesize them, including the exploitation of enzymes specific for the D-configuration of amino acid derivatives [1–4]. Among them, D-stereoselective amino acid amide amidohydrolase, called D-amino acid amidase, hydrolyzing the carboxyl amide bonds of D-amino acid amides to form D-amino acids and ammonia, are useful for the production of enantiomerically pure D-amino acids from the corresponding racemic amino acid amides, which are readily synthesized from aldehyde, hydrogen cyanide, and ammonium, via aminonitrile as an intermediate [1]. If the enzyme is used together with amino acid amide racemase catalyzing the racemization of the remaining L-amino acid amides, the racemic amino acid amides could be completely hydrolyzed to form D-amino acids [5–7].

The D-amino acid amidases known to date can be classified into four groups based on their primary structures and characteristics. D-Amino acid amidase, $DaaA_{Oa}$, from *Ochrobactrum anthropi* SV3 [8,9] and D-aminopeptidase, DAP, from *O. anthropi* C1-38 [10,11] are active site serine hydrolases, which belong to the penicillin-recognizing protein family together with DD-carboxypeptidases involved in the biosynthesis of bacterial cell wall peptidoglycan, class C β-lactamases for resistance against β-lactam antibiotics, and D-stereospecific endopeptidases [12–14] (Group 1). A thermostable D-alanine amidase from *Brevibacillus borstelensis* BCS-1 [15] is homologous to *Bacillus subtilis* D-aminopeptidase, DppA, which is a Zn^{2+}-dependent self-compartmentalizing protease composed of 10 subunits [16] (Group 2). *B. borstelensis* BCS-1 produces another D-amino acid amidase called thermostable D-methionine amidase after the late-log phase of growth [17]. Although gene cloning of the enzyme has not been done yet, the N-terminal amino-acid sequence of the enzyme purified from the parent strain was found to show significant similarity to that of the thermostable D-alanine amidase and of DppA, indicating that the thermostable D-methionine amidase can be classified into Group 2. D-Amino acid amidases from *Variovorax paradoxus* strain 19-3 [18] and *Delftia acidovorans* strain 16 [19] acting on D-*tert*-leucinamide, a carboxamide of non-proteinogenic D-amino acid with a bulky *tert*-butyl side chain, show sequence similarity with amidases involved in the metabolism of nitrile with a genetically linked nitrile hydratase in several microorganisms (Group 3). A D-alaninamide-specific amide hydrolase, called D-amidase, from *Arthrobacter* sp. NJ-26 has been reported to be useful for the production of D-alanine from racemic alaninamide [20]. Although the primary structure of the enzyme

* Corresponding author. Tel.: +81 766 56 7500; fax: +81 766 56 2498.
E-mail address: asano@pu-toyama.ac.jp (Y. Asano).

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doi:10.1016/j.enzmictec.2008.03.008

has not been revealed yet, comparison of the characteristics of the enzyme with those of the other reported D-amino acid amidases indicates that the NJ-26 D-amidase does not belong to the above three groups (Group 4).

It is worthwhile to prepare a collection of various D-amino acid amidases for the enzymatic synthesis of a variety of D-amino acids. In order to find a new D-amino acid amidase, we screened for microorganisms with D-phenylalaninamide as a substrate. In this paper, we describe the gene cloning and characterization of a new D-amino acid amidase from *Brevibacterium iodinum* TPU 5850. This enzyme, named $DaaA_{Bi}$, is not homologous to any of the D-amino acid amidases mentioned above, but unexpectedly is homologous to hypothetical class A β-lactamases from several bacteria. $DaaA_{Bi}$ was found to be a novel D-amino acid amidase exhibiting strict D-stereoselective hydrolytic activity towards a broad range of amino acid amides.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

B. iodinum TPU 5850 (TPU: stock cultures of Toyama Prefectural University) was selected as a microorganism capable of hydrolyzing D-phenylalaninamide and used as the source of chromosomal DNA. *E. coli* strains XL1-Blue MR (*Δ*(*mcr*A)183, *Δ*(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*) and JM109 (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *Δ* (*lac*-*pro*AB)/F′ [*tra*D36, *pro*AB$^+$, *lacI*^{q}, *lacZ*ΔM15]) were used as hosts for the recombinant plasmids. Plasmids pBluescriptII SK(−) (Toyobo, Osaka, Japan) and pUC19 (Takara Bio Inc. Shiga, Japan) were used as cloning vectors. *B. iodinum* TPU 5850 was grown in TGY medium containing 5 g of Bacto™ tryptone (Difco), 5 g of yeast extract, 1 g of glucose, and 1 g of K_2HPO_4 in 1 L of distilled water, pH 7.0. Recombinant *E. coli* was cultured in Luria-Bertani medium [21] containing ampicillin (80 μg mL^{-1}). To induce expression of the gene under the control of the *lac* promoter, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM.

2.2. Cloning of the B. iodinum TPU 5850 D-amino acid amidase gene ($daaA_{Bi}$)

For routine work with recombinant DNA, established protocols were used [21]. Restriction endonucleases were purchased from Takara Bio Inc. Alkaline phosphatase from shrimp was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Chromosomal DNA was prepared from *B. iodinum* TPU 5850 by the method of Misawa et al. [22] and partially digested with *Sau*3AI. The resulting fragments, 2–9 kbp in size, were isolated from 0.7% (w/v) agarose gel by use of a QIAquick™ gel extraction kit from QIAGEN (Tokyo, Japan) and ligated into *Bam*HI-digested and alkaline phosphatase-treated pBluescriptII SK(−) using Ligation Kit ver. 2 (Takara Bio Inc.). *E. coli* XL1-Blue MR was transformed with the recombinant plasmid DNA and ampicillin-resistant transformants were selected. Visualization of the D-amino acid amidase activity expressed in the transformants was carried out using a D-amino-acid oxidase and peroxidase-coupled reaction as described previously [9]. A colony that developed a red color was picked as a positive clone. The *E. coli* transformant carried a 7.2-kb plasmid, designated pBio1.

2.3. DNA sequence analysis

An automatic plasmid isolation system, PI-100 from Kurabo (Osaka, Japan), was used to prepare the double-stranded DNAs for sequencing. The plasmid pBio1 was used as a sequencing template. Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method [23]. Sequencing reactions were carried out with a BigDye^R Terminator v3.1 cycle sequencing kit (Applied Biosystems Japan Ltd., Tokyo, Japan) and the reaction mixtures were run on a Genetic analyzer ABI PRISM 310 system (Applied Biosystems Japan Ltd.). Both strands of DNA were completely sequenced. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB233405. Amino-acid sequences were compared with the BLAST program [24].

2.4. Expression of the $daaA_{Bi}$ gene in E. coli

A DNA fragment of ORF2, which was later defined as $daaA_{Bi}$ was obtained by PCR using the Expand high fidelity^{PLUS} PCR system from Roche Diagnostics GmbH. The reaction mixture for the PCR contained 50 μL of Expand high fidelity^{PLUS} buffer with 1.5 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, a sense and an antisense primer each at a concentration of 1 μM, 2.5 U of Expand high fidelity^{PLUS} PCR system enzyme mix, and 200 ng of plasmid pBio1 as a template. Thirty cycles were performed, each consisting of a denaturing step at 94 °C for 30 s (first cycle 2 min 30 s), an anealing step at 55 °C for 30 s and an elongation step at 72 °C for 2 min. The sense primer contained an *Hin*dIII-recognition site (underlined sequence), a ribosome-binding site (double underlined sequence), a TAG stop codon (lowercase letters) in-frame with the *lacZ* gene in pUC19, and spanned positions 1147–1174 in the sequence with Genbank accession number AB233405. The antisense primer contained an *Xba*I site (underlined sequence) and corresponded to the sequence from 2007 to 2038. The two primers were as follows: sense primer, 5′ − ACGAAAAAGCTTTAAGGAGGAAtagATGATGAGCGATTTGAAATTCGATGGAC − 3′; antisense primer, 5′-TGGGTGTCCGTCTAGATCGTATCGGACATCGC-3′. The amplified PCR product was digested with *Hin*dIII and *Xba*I, separated by agarose-gel electrophoresis, and then purified with the QIAquick™ gel extraction kit. The amplified DNA was inserted downstream of the *lac* promoter in pUC19, yielding pDaaABi, and then used to transform *E. coli* JM109 cells.

2.5. Purification of $DaaA_{Bi}$ from the E. coli transformant

E. coli XL1-Blue MR harboring pBio1 was subcultured at 37 °C for 12 h in a test tube containing 5 mL of Luria-Bertani medium supplemented with ampicillin. The subculture (5 mL) was then inoculated into a 2-L Erlenmeyer flask containing 500 mL of Luria-Bertani medium supplemented with ampicillin and isopropyl-β-D-thiogalactopyranoside. After a 16-h incubation at 37 °C with rotary shaking, the cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C and washed with 0.9% (w/v) NaCl. All the purification procedures were performed at a temperature lower than 4 °C. The buffer used was potassium phosphate (pH 7.0). The protein content of the eluate from column chromatography was monitored by measuring absorbance at 280 nm. Washed cells from the 2.5-L culture were suspended in 100 mM buffer and disrupted by sonication with an ultrasonic oscillator (19 kHz insonator model 201 M: Kubota, Japan). For the removal of intact cells and cell debris, the sonicate was centrifuged at 15,000 × *g* for 10 min at 4 °C, and the resulting supernatant was used as the cell-free extract. To the cell-free extract was added 5% protamine sulfate, at a concentration of 4.7 mg of protamine sulfate to 100 mg of protein. After stirring for 10 min, the precipitate formed was removed by centrifugation at 15,000 × *g* for 10 min at 4 °C. The resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 30–60% saturation was collected by centrifugation and dissolved in 20 mM buffer. The resulting enzyme solution was dialyzed against 15 L of the same buffer for 24 h. The dialyzed solution was applied to a column (Ø 2.4 cm × 13.5 cm) of DEAE-Toyopearl 650 M (Tosoh Corp., Tokyo, Japan) previously 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 (0–0.5 M) in 20 mM buffer. To the active fractions was added ammonium sulfate to 30% saturation. The enzyme solution was applied to a column (Ø 1.4 cm × 7.5 cm) of Butyl-Toyopearl 650 M (Tosoh Corp.) previously equilibrated with 20 mM buffer containing ammonium sulfate to 30% saturation. The active fractions were eluted with a linear gradient of ammonium sulfate (30–0% saturation) in 20 mM buffer. The active fractions were combined and dialyzed against 15 L of 20 mM buffer for 24 h. The enzyme solution was applied to a MonoQ HR 10/10 column (Amersham Biosciences K.K., Tokyo, Japan) previously equilibrated with 20 mM buffer. After the column had been washed with 30 mL of 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in 20 mM buffer using the Äkta-FPLC system (Amersham Biosciences K.K.). The active fractions were combined, dialyzed against 10 L of 20 mM buffer for 24 h, and used for characterization.

2.6. Enzyme assay

Activity of $DaaA_{Bi}$ was assayed under standard conditions with D-phenylalaninamide as a substrate. The reaction mixture (0.5 mL) contained 50 μmol potassium phosphate buffer (pH 7.0), 25 μmol D-phenylalaninamide and an appropriate amount of the enzyme. The reaction was performed at 30 °C for 10 min and stopped by the addition of 0.1 mL of 2 M $HClO_4$. The amount of D-phenylalanine formed was determined with an HPLC apparatus equipped with a COSMOSIL $5C_{18}$-MS-II column (Ø 0.46 cm × 15 cm) (Nacalai tesque, Kyoto, Japan) at a flow rate of 0.7 mL min^{-1}, using the solvent system 5 mM H_3PO_4/methanol (4:1, v/v). Absorbance of the eluate was monitored at 254 nm. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol D-phenylalanine min^{-1} from D-phenylalaninamide under the above conditions. Enzyme activity toward other amino-acid amides and peptides was determined with an HPLC apparatus equipped with a Sumichiral OA-5000 column (Ø 0.46 cm × 15 cm) (Sumika Chemical Analysis Service, Osaka, Japan) at a flow rate of 1.0 mL min^{-1}, using as a solvent system, 2 mM $CuSO_4$ or 2 mM $CuSO_4$/2-propanol (19:1, v/v). The absorbance of the eluate was monitored at 254 nm. The hydrolysis of β-lactam compounds was monitored by loss of absorbance as follows: benzylpenicillin ($\Delta\varepsilon_{232}$ = 940 M^{-1} cm^{-1}), ampicillin ($\Delta\varepsilon_{235}$ = 820 M^{-1} cm^{-1}), and cefotaxime ($\Delta\varepsilon_{262}$ = 7250 M^{-1} cm^{-1}). Protein concentrations were determined by the method of Bradford [25] using BSA as a standard. Data are means of three experiments and standard errors were below 5%.

2.7. Analytical measurements

To estimate the M_r of the enzyme, the sample (15 μg) was subjected to HPLC on a Superdex 200 HR10/30 column (Amersham Biosciences K.K.) at a flow rate of 0.4 mL min^{-1} with 20 mM potassium phosphate (pH 7.0) containing 150 mM NaCl at