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Characterization of an enantioselective amidase from *Cupriavidus* sp. KNK-J915 (FERM BP-10739) useful for enzymatic resolution of racemic 3-piperidinecarboxamide

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ABSTRACT

A novel amidase (*Cs*AM) acting on (*R*,*S*)-*N*-benzyl-3-piperidinecarboxamide was purified from *Cupriavidus* sp. KNK-J915 (FERM BP-10739) and characterized. The enzyme acts on (*R*,*S*)-*N*-benzyl-3-piperidinecarboxamide *S*-selectively to yield (*R*)-*N*-benzyl-3-piperidinecarboxamide. Analytical gel filtration column chromatography and SDS-PAGE revealed that the enzyme is a tetramer with a subunit of approximately 47 kDa. It has a broad substrate spectrum against nitrogen-containing heterocyclic amides. Its optimal pH and temperature are 8.0–9.0 and 50 °C, respectively. The *Cs*AM gene was cloned and sequenced, and it was found to comprise 1341 bp and encode a polypeptide of 46,388 Da. The deduced amino acid sequence exhibited 78% identity to that of a putative amidase (*Cn*AM) from *Cupriavidus necator* JMP134. The cultured cells of recombinant *Escherichia coli* producing *Cn*AM could be used for the *S*-selective hydrolysis of (*R*,*S*)-*N*-benzyl-3-piperidinecarboxamide but could not be used for the *S*-selective hydrolysis of (*R*,*S*)-3-piperidinecarboxamide because of its very low level of selectivity. In contrast, the cultured cells of recombinant *E. coli* producing *Cs*AM could hydrolyze both (*R*,*S*)-*N*-benzyl-3-piperidinecarboxamide and (*R*,*S*)-3-piperidinecarboxamide with high *S*-selectivity.

1. Introduction

Enzymatic or chemoenzymatic procedures including kinetic resolution or desymmetrization using amidase have been reported for the preparation of optically active amides, acids, amino acids, and amines [1–4]. Therefore, amidase is expected to be a promising biocatalyst for the synthesis of various chiral compounds.

(*R*)-3-Aminopiperidine is a common intermediate in the synthesis of dipeptidyl peptidase-4 (DPP-4) inhibitors, which comprise a class of oral antidiabetic drugs [5,6]. Therefore, the establishment of the efficient synthesis of (*R*)-3-aminopiperidine on an industrial scale is required. (*R*)-3-piperidinecarboxamide with high optical purity is a valuable synthetic intermediate because it is expected to be converted to (*R*)-3-aminopiperidine using the Hofmann rearrangement [7]. Therefore, we sought to develop a novel biocatalytic process for obtaining (*R*)-3-piperidinecarboxamide in high enantiomeric excess using an *S*-selective amidase. An *R*-selective amidase from *Pseudomonas* sp. that could act on (*R*,*S*)-3-piperidinecarboxamide has been reported, but the enantiomeric excess of the corresponding (*S*)-3-piperidinecarboxamide or (*R*)-3-piperidinecarboxylic acid was not described [8]. In this study, we screened for microorganisms that can selectively hydrolyze the *S*-enantiomer in (*R*,*S*)-*N*-benzyl-3-piperidinecarboxamide (BNPD) (Fig. 1) and identified the amidase activity in *Cupriavidus* sp. KNK-J915 (FERM BP-10739). The amidase purified from cells of the strain *S*-selectively hydrolyzed (*R*,*S*)-BNPD to form (*R*)-BNPD, and the enzyme was referred to as *Cs*AM. *Cs*AM was characterized and found to be a novel amidase with unique substrate specificity. The gene encoding *Cs*AM was isolated, sequenced, and expressed in *Escherichia coli*.

2. Materials and methods

2.1. Chemicals

BNPD, (*R*)-BNPD, (*R*)-*N*-benzyloxycarbonyl-3-piperidinecarboxamide, (*R*,*S*)-*N*-benzyl-3-piperidinecarboxylic acid (BNPA), (*R*,*S*)-piperidine-2-carboxamide, (*R*,*S*)-indoline-2-carboxylic acid amide, DL-phenylalanine amide, DL-β-phenylalanine amide, (*R*,*S*)-mandelic acid amide, and (*R*,*S*)-2-phenylpropionic acid amide were prepared according to existing methods. Piperidine-4-carboxamide was purchased from Sigma–Aldrich Co. LLC.

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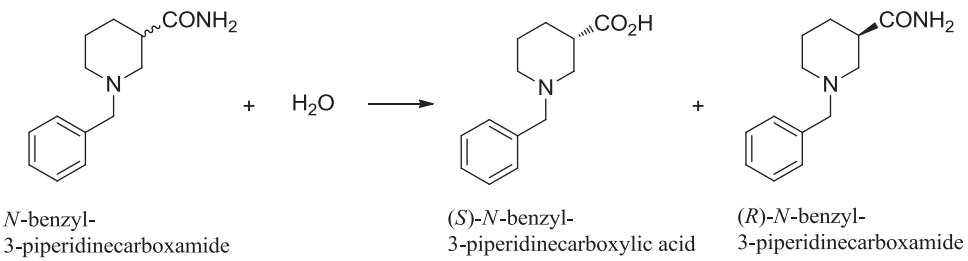

Fig. 1. Stereoselective hydrolysis of *N*-benzyl-3-piperidinecarboxamide.

(*R*,*S*)-3-Piperidinecarboxamide (NPD), and (*R*,*S*)-3-piperidinecarboxylic acid (NPA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals used in this study were of analytical grade and were commercially available.

2.2. Microorganisms, culture conditions, and plasmids

Microorganisms were obtained from our laboratory collection. *Cupriavidus* sp. KNK-J915 (FERM BP-10739) was selected as a microorganism capable of hydrolyzing (*R*,*S*)-BNPD with *S*-selectivity and used as a source of enzyme and chromosomal DNA. The medium used for the screening consisted of 1.0% glycerol, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 0.1% isovaleronitrile (pH 7.0). The CM medium consisted of 1.0% meat extract, 1.0% peptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0). The medium P consisted of 2.0% glycerol, 1.0% meat extract, 1.0% peptone, and 0.5% yeast extract (pH 7.0). *E. coli* HB101 was used as the host cell for gene cloning and expression. Transformed *E. coli* was cultured at 30 °C in 2-YT medium containing 1.6% Bacto™ Tryptone, 1.0% Bacto™ Yeast Extract, and 0.5% NaCl (pH 7.0). When necessary, ampicillin (0.1 mg/mL) was added to the medium. Plasmid pUCNT was prepared from pUC19 and pTrc99A as previously described [9].

2.3. Screening method for S-selective BNPD-hydrolyzing microorganisms

In the screening experiments, each strain was inoculated into 8 mL of medium in a test tube (24 mm i.d. × 200 mm), followed by incubation at 30 °C with reciprocal shaking for 3 days. Microorganisms were harvested by centrifugation. The cells from 8 mL of culture broth were suspended in 1 mL of 100 mM potassium phosphate (pH 7.0). The resulting cell suspensions (0.1 mL each) were separately mixed with 0.1 mL of (*R*,*S*)-BNPD solution [9.16 mM, solved in 100 mM potassium phosphate (pH 7.0)], and the reaction mixtures were stirred at 30 °C for 24 h. After the reaction, aliquots of the reaction mixtures were withdrawn and analyzed by high-performance liquid chromatography (HPLC) to determine the conversion degree (%) of the substrate to the product in the reaction solution and the optical purity (% e.e.).

The value of enantioselectivity (*E*-value) was calculated from the conversion degree (c) and the enantiomeric excess of the substrate (ees) was calculated according to a previously described method [10] (Eq. (1)).

$$E = \frac{ln(1-c)(1-ees)}{ln(1-c)(1+ees)} \quad (1)$$

2.4. Enzyme assay

During the purification and characterization of amidase from *Cupriavidus* sp. KNK-J915, an enzyme assay was performed with (*R*,*S*)-BNPD as a substrate. The standard reaction mixture (0.2 mL) contained 100 mM potassium phosphate buffer (pH 7.0), 45.8 mM BNPD, and an appropriate amount of the enzyme. After the reaction was performed at 30 °C for 0.5–1 h, the amount of BNPA was determined using HPLC. One unit of the enzyme was defined as the amount catalyzing the formation of 1 μmol of BNPA per minute under the aforementioned condition. Protein content was determined by the Bradford method [11] with BSA as a standard using a kit from Bio-Rad Laboratories Ltd. (Tokyo, Japan).

In the characterization of recombinant *Cs*AM and *Cn*AM from *E. coli* transformant experiments, each strain was inoculated into 5 mL of 2-YT medium in a test tube (24 mm i.d. × 200 mm), followed by incubation at 30 °C with reciprocal shaking for 24 h. Microorganisms were harvested by centrifugation. The cells from 5 mL of culture broth were suspended in 2.5 mL of 100 mM potassium phosphate (pH 7.0). The resulting cell suspensions (0.1 mL each) were separately mixed with 0.1 mL of (*R*,*S*)-BNPD solution [91.6 mM, solved in 100 mM potassium phosphate (pH 7.0)] or 0.1 mL of (*R*,*S*)-NPD solution [156 mM, solved in 100 mM potassium phosphate (pH 7.0)], and the reaction mixtures were stirred at 30 °C for 2 h. After the reaction, aliquots of the reaction mixtures were withdrawn and analyzed by HPLC to determine the conversion degree (%) of the substrate to the product in the reaction solution and optical purity (% e.e.).

Enzyme activity toward other amides [each concentration was 1.0%, solved in 100 mM potassium phosphate (pH 7.0)] was determined by measuring the formation of ammonia using Ammonia Test WAKO (Wako Pure Chemicals, Osaka, Japan). One unit of the enzyme was defined as the amount catalyzing the formation of 1 μmol of ammonia per minute.

2.5. Analytical methods

The degree of conversion of BNPD to BNPA was analyzed by reverse-phase HPLC using a Shimadzu LC-VP system (Shimadzu, Kyoto, Japan) equipped with a YMC-A303 column (4.6 mm × 250 mm; YMC, Kyoto, Japan). HPLC was conducted using acetonitrile:water (1:9 by vol., pH 2.5, adjusted with phosphoric acid) as the mobile phase, a flow rate of 1.0 mL/min, a column temperature of 30 °C, and ultraviolet (UV) detection at 210 nm. The optical purity of BNPD was determined by reverse-phase HPLC using a Shimadzu LC-VP system equipped with a CHIRALPACK AD-RH column (4.6 mm × 150 mm; Daicel, Osaka, Japan). HPLC was conducted using acetonitrile:20 mM potassium phosphate buffer (pH 8.0; 3:7 by vol.) as the mobile phase, a flow rate of 0.5 mL/min, a column temperature of 30 °C, and UV detection at 210 nm.

NPD and NPA in the reaction solution were derivatized using carbobenzoxy chloride and the obtained derivatives were analyzed. The derivatization procedure using carbobenzoxy chloride was as follows. The saturated aqueous $NaHCO_3$ (200 mg) was added to the reaction mixture (100 mg). The mixture was concentrated under reduced pressure to remove NH_3. Then, THF (200 mg) and carbobenzoxy chloride (25 mg) were added to the concentrated solution. The mixture was stirred for 10 min, and then diluted with water. The diluted solution was used as NPD and NPA