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Discovery of D-Stereospecific Dipeptidase from Thermophilic *Bacillus* sp. BCS-1 and Its Application for Synthesis of D-Amino Acid-Containing Peptide

BAEK, DAE-HEOUN, SEOK-JOON KWON, JIN-SEO PARK, SEUNG-GOO LEE, TAE-ICK MHEEN, AND MOON-HEE SUNG*

Microbial Conversion Research Unit, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Taejon 305-600, Korea

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Abstract A thermophilic bacterium producing D-stereospecific dipeptidase was isolated from Korean soil samples. The enzyme hydrolyzed the peptide bond between D-alanyl-D-alanine (D-Ala-D-Ala). The isolated bacterial strain was rod shaped, gram-positive, motile, and formed an endospore. Morphological and physiological characteristics suggested this microorganism a thermophilic *Bacillus* species, and was named as *Bacillus* sp. BCS-1. The production of D-stereospecific dipeptidase was growth-associated and optimal at 55°C. The enzyme was applied for the synthesis of D-amino acid-containing peptide, *N*-benzyloxycarbonyl-L-aspartyl-D-alanine benzyl ester (Z-L-Asp-D-AlaOBzl), as a model reaction. A thermodynamically controlled synthesis of Z-L-Asp-D-AlaOBzl was achieved in an organic solvent.

Key words: Screening, thermophilic *Bacillus*, D-stereospecific dipeptidase, enzymatic synthesis, D-amino acid-containing peptide

D-Amino acids occur in bacterial cell wall peptidoglycan [12] and in higher plants [11]. In addition, several biologically active peptides, including neuropeptides [10], synthetic vaccines [13], peptide sweeteners [9], antibiotics [8], and other hormones [5] contain D-amino acid residues. Hence, it is important to screen useful thermostable enzymes for the synthesis of D-amino acid-containing peptides.

Many researchers have explored D-stereospecific enzymes hydrolyzing D-amino acid derivatives [2-4], and so-far isolated D-stereospecific enzymes from *Ochrobactrum anthropi* [2], *Bacillus cereus* [4], *Nocardia orientalis* [16], and *Enterococcus faecium* [18]. All of these microorganisms are mesophiles, and their enzymes are the most active at 37°C or below. However, D-stereospecific dipeptidase has not yet been discovered in thermophiles.

In order to screen thermostable D-stereospecific dipeptidases, D-alanyl-D-alanine (D-Ala-D-Ala) was used as the substrate for the enzyme. Fifteen hundred thermophiles in Korean soil were tested, and one thermophile with D-stereospecific dipeptidase activity was isolated. This paper describes the isolation and taxonomic identification of a novel D-stereospecific dipeptidase-producing thermophile isolated from Korean soil samples, and its application in the synthesis of D-amino acid-containing peptides.

Materials and Methods

Materials

D-Alanyl-D-alanine (D-Ala-D-Ala), D-amino acid oxidase (D-AAO, from porcine kidneys), 4-aminoantipyrine (4-AP), *N*-ethyl-N-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (USA). *N*-Benzyloxycarbonyl-L-aspartic acid (Z-L-Asp-OH) and D-alanine benzyl ester (D-AlaOBzl) were purchased from Bachem Co. (Bubendorf, Switzerland). Peroxidase was purchased from Wako Pure Chem. (Japan).

Screening of D-Stereospecific Dipeptidase-Producing Thermophile

The 1,500 thermophiles isolated from Korean soil were cultivated in Luria-Bertani (LB) medium (pH 7.2) at 55°C for about 12 h in a rotary shaking incubator. The harvested cells were disrupted by sonication at 20 kHz for 5 min, and centrifuged at 18,000 ×g for 30 min. The supernatant was dialyzed against 100 mM Tris-HCl buffer (pH 8.0) containing 0.3 mM PMSF and β-mercaptoethanol at 4°C overnight.

*Corresponding author
Phone: 82-42-860-4372; Fax: 82-42-860-4595;
E-mail: smoonhee@mail.kribb.re.kr

The cell-free extracts obtained were tested for D-stereospecific dipeptidase activity.

Identification and Characterization of Isolated Strain BCS-1

Taxonomical and biological characteristics of strain BCS-1 were investigated using the procedures described in before [7, 15, 17].

Enzyme Assay

D-Stereospecific dipeptidase activity was assayed at 55°C by measuring the production of D-alanine from D-alanyl-D-alanine [2]. The reaction mixture contained 0.3 mM PMSF, 5 mM β-mercaptoethanol, 5 mM D-Ala-D-Ala, and 20 μl of cell-free extract in 0.5 ml of 50 mM Tris-HCl (pH 8.0). The reaction was carried out at 55°C for 30 min, and terminated by boiling for 3 min. The reaction sample was added to a standard reaction mixture (1.5 ml) containing 50 mM Tris-HCl (pH 8.0), 0.15 units of D-AAO, 12 mM of TOOS, 0.53 mM of 4-AP, and 14 units of peroxidase, and the mixture was incubated at 30°C for 30 min while shaking, unless otherwise specified. The hydrogen peroxide formed from D-alanine by the action of D-AAO was determined by oxidative coupling with 4-AP and TOOS (used as a substitute for phenol) in the presence of peroxidase [1]. The formation of quinone-imine dye was measured at 555 nm and quantified by the standard curve obtained from authentic D-alanine. One unit of enzyme activity was defined as the amount of enzyme which catalyzed degradation of 1 μmol of D-Ala-D-Ala per min. The protein concentration was determined by using bovine serum albumin as the standard [6].

Enzymatic Synthesis of Z-L-Asp-D-AlaOBzl

The D-stereospecific dipeptidase from *Bacillus* sp. BCS-1 was partially purified by ammonium sulfate fractionation (30-80%), dialysis, and column chromatography using Toyopearl QAE, Phenyl Sepharose, and Resource Q. The partially purified enzyme preparation was then suspended in 50 mM MES-NaOH buffer containing 0.3 mM PMSF and 10 mM Z-L-AspOH, and the pH was adjusted to 6.0 with NaOH. The enzyme solution was lyophilized. Z-L-AspOH and D-AlaOBzl·*p*-tosylate were used as acyl donor and acyl acceptor, respectively. The reaction was initiated by adding the lyophilized enzyme powder (0.3 hydrolytic units/ml) to organic solvents containing 30 mM D-AlaOBzl·*p*-tosylate, and was carried out with vigorous shaking (175 rpm) at 40°C. Aliquots (50 μl) were withdrawn from the reaction mixture, and mixed with 1 N HCl to stop any further enzymatic reaction. The samples were subsequently analyzed by HPLC using an octadecylsilica column at 254 nm. One of the elution solvents was solvent A (0.1% (v/v) aqueous TFA), and the other was solvent B (CH_3CN containing 0.1% (v/v) TFA). The following gradient elution was used; 90:10 (A:B) to 0:100 (A:B) for 20 min.

RESULTS

Screening of D-Stereospecific Dipeptidase-Producing Thermophiles

The D-stereospecific dipeptidase-producing thermophiles were screened by the oxidative coupling of D-alanine liberated from D-Ala-D-Ala. Of the 1,500 thermophiles isolated from the Korean soild, one strain (BCS-1) showed significant activity (0.002 units/mg protein).

Identification and Characterization of the Isolated Strain No. BCS-1

Morphological and physiological characteristics of the strain BCS-1 were investigated and they were compared with the type strains *B. stearothermophilus* and *B. brevis* (Table 1). From the results, the isolated strain BCS-1 was identified as the *Bacillus* species.

Growth and Enzyme Production of Thermophilic *Bacillus* sp. BCS-1

When grown at various temperatures, the optimum growth temperature of strain BCS-1 was found to be 55°C. As shown in Fig. 1, the production of D-stereospecific dipeptidase was growth-associated, and its maximum activity (0.0025 units/mg) was at the end of the growth phase (6 h).

Table 1. Morphological and biological characteristics of strain BCS-1.

Characteristics	Strain BCS-1	*B. stearothermophilus*	*B. brevis*
Cell shape	Rod	Rod	Rod
Gram staining	+	+	v
Endospore	+	+	+
Motility	+	v	+
Growth temperature	30~58°C	40~65°C	30~55°C
Voges-Proskauer	+	-	-
Nitrate reduction	+	v	v
Utilization of			
Glucose	-	+	v
Mannitol	-	v	v
Arabinose	-	v	-
Production of			
β-Galactosidase	-	-	-
Tryptophan deaminase	+	-	-
Catalase	+	v	+
Gelatinase	+	+	+
Growth on pH 5.7	w	v	v
7% NaCl	-	-	-
Anaerobic growth	-	-	-

+, Positive reaction; -, negative reaction; w, weak; v, variable.

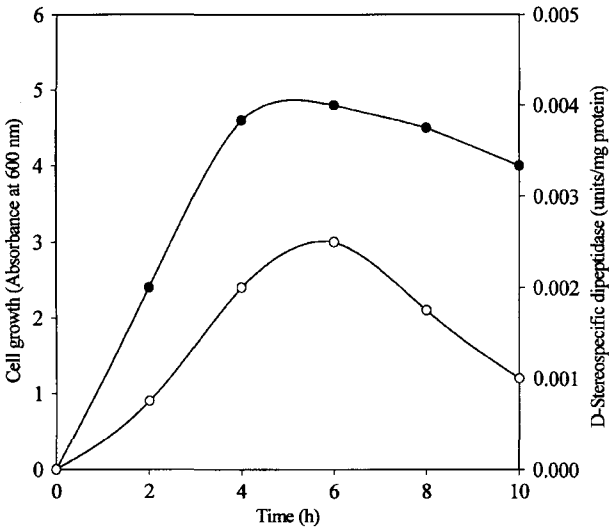

Fig. 1. Time course of D-stereospecific dipeptidase production by *Bacillus* sp. BCS-1 in an LB medium.
Cultivation was carried out at 55°C for 10 h in 100 ml of the medium in a 1 liter baffled-flask. Symbols: ●, cell growth; ○, enzyme activity.

Synthesis of D-Amino Acid-Containing Peptide Using D-Stereospecific Dipeptidase

The D-stereospecific dipeptidase was partially purified and applied to the synthesis of a D-amino acid-containing peptide. The enzymatic synthesis of Z-L-Asp-D-AlaOBzl was carried out as a model reaction, since this high potency peptide sweetener has the structural backbone of L-Asp-D-Ala [9]. A thermodynamically controlled synthesis was performed via the D-stereospecific dipeptidase-catalyzed condensation of Z-AspOH and D-AlaOBzl. This type of synthesis has an advantage when compared to a kinetically controlled synthesis, since it does not require any protection of the Z-AspOH carboxyl group. The effects of various organic solvents on the peptide synthesis were investigated. As shown in Table 2, the enzyme preferred water immiscible organic solvents for the peptide synthesis to water miscible organic solvents. Among the various solvents, ethylether was found to be the most suitable. The reaction product (Z-L-Asp-D-AlaOBzl) was collected from HPLC and identified by electrospray ionization mass spectrometry (ESI-MS, Z-L-Asp-D-AlaOBzl: m/z 429 $(M+H)^+$, 451 $(M+Na)^+$.

Table 2. Effects of various organic solvents on the synthesis of Z-L-Asp-D-AlaOBzl by *Bacillus* sp. BCS-1 D-stereospecific dipeptidase.

Solvents	Conversion yield (%)[a]
Dimethyl sulfoxide	0
Acetonitrile	0
Dimethyl formamide	0
Tetrahydrofuran	0
Methyl acetate	16.1
Ethyl acetate	13.2
Butyl acetate	20.6
Ethyl ether	26.7
Isooctane	13.2
Hexane	13.6

[a]Conversion yield means % conversion of 10 mM Z-L-AspOH into Z-L-Asp-D-AlaOBzl after 8 h. The reaction conditions are described in Materials and Methods.

Discussion

This paper described the identification of a new D-stereospecific dipeptidase from thermophilic *Bacillus* sp. BCS-1. The main focus was on the screening of thermostable enzymes as potential biocatalysts, because thermostable enzymes produced by thermophiles (although not all) were intrinsically thermostable, and this enhanced stability was also demonstrated against the action of other protein denaturants such as detergents and organic solvents [14]. To the best of our knowledge, there had been no previous report on D-stereospecific dipeptidase from thermophiles which catalyzed the hydrolysis of D-Ala-D-Ala. Accordingly, it is proposed that D-stereospecific dipeptidase from thermophilic *Bacillus* sp. BCS-1 is a new enzyme; however, the action mechanism and stability of the enzyme remain to be clarified. In addition, D-stereospecific dipeptidase was successfully applied to the synthesis of a D-amino acid-containing peptide (Z-L-Asp-D-AlaOBzl). Therefore, D-stereospecific dipeptidase may have potential application in the synthesis of other useful D-amino acid-containing peptides.

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