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Research paper

Aminolytic reaction catalyzed by D-stereospecific amidohydrolases from *Streptomyces* spp

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ABSTRACT

From investigation of 2000 soil isolates, we identified two serine-type amidohydrolases that can hydrolyze D-aminoacyl derivatives from the culture supernatant of *Streptomyces* species 82F2 and 83D12. The enzymes, redesignated as 82F2-DAP and 83D12-DAP, were purified for homogeneity and characterized. Each enzyme had molecular mass of approximately 40 kDa, and each showed moderate stability with respect to temperature and pH. Among hydrolytic activities toward D-aminoacyl-pNAs, the enzymes showed strict specificity toward D-Phe-pNA, but showed broad specificity toward D-aminoacyl esters. The specific activity for D-Phe-pNA hydrolysis of 82F2-DAP was ten-fold higher than that of 83D12-DAP. As a second function, each enzyme showed peptide bond formation activity by its function of aminolysis reaction. Based on results of D-Phe–D-Phe synthesis under various conditions, we propose a reaction mechanism for D-Phe–D-Phe production. Furthermore, the enzymes exhibited peptide elongation activity, producing oligo homopeptide in a one-pot reaction. We cloned the genes encoding each enzyme, which revealed that the primary structure of each enzyme showed 30–60% identity with those of peptidases belonging to the clan SE, S12 peptidase family categorized as serine peptidase with D-stereospecificity.

1. Introduction

Peptides in nature incorporating D-amino-acids are useful as antimicrobial compounds and signal molecules. They are expected to widen and extend the functional variety and applications of peptides [1–5]. Although D-amino-acid-containing peptides have been produced using organic synthesis [6,7], the complicated reaction steps and the racemization of amino acids are persistent difficulties associated with their organic synthesis. Therefore, enzyme-catalyzed peptide synthesis is widely regarded as an alternative means of chemical synthesis. Recent reports have described enzymatic syntheses using peptidase [8,9], esterase [10], aminoacyltransferase [11], and D-alanine–D-alanine ligase [12]. Among them, aminolysis reactions, reactions of an amine with a carboxylic acid derivative to form an amide, of serine amidohydrolases containing peptidases, esterases, and aminoacyl transferases have increasingly attracted attention as tools for peptide synthesis. Recent reports describe enzymatic synthesis of β-amino acid containing peptides [13,14], diverse prolyl peptides [15], and D-alanine oligopeptides [8]. Their simple kinetics and the obviation of expensive additives make this peptide synthetic system easy to use [16,17].

In this study, we identified D-stereospecific amidohydrolases (D-aminopeptidase) in the culture supernatant of *Streptomyces* spp. 82F2 and 83D12, the activities of which were inhibited by phenylmethylsulfonyl fluoride (PMSF). The enzyme is of particular interest as a useful tool for synthesis of various peptides incorporating D-amino-acid because it has a function of aminolysis reaction. This report describes the mechanism of peptide bond formation, differences in properties of obtained enzymes, and sequence analysis data.

2. Materials and methods

2.1. Materials and bacterial strain

Peptides, aminoacyl *p*-nitroanilides (pNAs), and other aminoacyl derivatives were purchased from Bachem AG, Aldrich Chemical Co. Inc., Sigma Chemical Co., Novabiochem Corp., and Wako Pure Chemical Industries, Ltd. *Streptomyces* spp. 82F2 and 83D12 were isolated from soil and identified using 16S ribosomal DNA analysis.

Abbreviations: 82F2-DAP, D-stereospecific amidohydrolases from *Streptomyces* sp. 82F2; 83D12-DAP, D-stereospecific amidohydrolases from *Streptomyces* sp. 83D12; PMSF, phenylmethylsulfonyl fluoride; pNA, *p*-nitroanilide; OBzl, benzyl ester; OMe, methyl ester; tBu, *tert*-butyl ester.

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2.2. Enzyme assay

For routine assay, the enzyme activity was determined using continuous spectrophotometric assay with a D-Phe-pNA substrate. In the assay, 0.1 ml of substrate solution (10 mM) was added to 0.9 ml of a mixture containing 200 mM Tris–malate (pH 6.5) and 10 μg/ml of enzyme at 25 °C. Increased absorption at 405 nm caused by the release of *p*-nitroaniline per minute was monitored continuously using a spectrophotometer (U2800; Hitachi, Ltd.). The initial rate was determined from a linear segment of the optical density profile (ε_{405} nm = 10,600 M^{-1} cm^{-1} [18]).

2.3. Purification of the enzymes from culture supernatant

Streptomyces spp. 82F2 and 83D12 were grown aerobically in 400 ml of culture medium containing 0.8% K_2HPO_4, 2.0% glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% polypeptone, and 0.5% yeast extract at 25 °C for 6 days. The culture supernatant was brought to 45% saturation using ammonium sulfate. Then the solution was loaded onto a column (Butyl-Sepharose Fast Flow; GE Healthcare) equilibrated with 100 mM Tris–HCl (pH 8.0) containing 45% ammonium sulfate. The column was washed with the same buffer containing 20% ammonium sulfate. Then the enzyme was eluted using 100 mM Tris–HCl. Fractions exhibiting high activity were pooled and then dialyzed against 20 mM Tris–HCl (pH 8.0). Next, the dialysate was loaded onto a spin column (Vivapure-Q; Sartorius AG) equilibrated with 20 mM Tris–HCl (pH 8.0). After washing with the same buffer, the bound protein was eluted with the same buffer containing 0.2 M NaCl. Fractions exhibiting high activity were pooled and then dialyzed against 20 mM sodium acetate (pH 5.5). The dialysate was loaded onto a spin column (Vivapure-S; Sartorius AG) equilibrated with 20 mM sodium acetate (pH 5.5). After washing with the same buffer containing 0.1 M NaCl, the bound protein was eluted with the same buffer containing 0.4 M NaCl. The homogeneity of purified proteins was confirmed using 12% SDS–PAGE under denaturing conditions [19].

2.4. Hydrolytic activity toward aminoacyl esters and peptides

The hydrolytic activity toward aminoacyl derivatives and peptides was determined through quantitative assay of amino acids, N-acetylated amino acids, and deesterified peptides derived from the hydrolyzed substrates. To 46 μl of 200 mM Tris–maleic acid (pH 6.5), 2 μl of an enzyme solution (0.05 or 0.2 mg ml^{-1}) and 2 μl of a substrate solution (0.5 M) were added. The reaction mixture was incubated at room temperature for 5 min. Then the reaction was stopped by the addition of 50 μl of 3% formic acid. The liberated amino acid was quantified using ultrahigh-performance liquid chromatography (UPLC)-electrospray ionization time-of-flight mass spectrometry (ESI–TOF MS) equipped with a C18 reverse-phase system (Acquity UPLC; Waters Corp.). The reaction mixture was diluted with 200-fold or 4000-fold volume of 0.1% formic acid and filtered. Then 5 μl of each sample was subjected to chromatography. Each sample was eluted with solvent A − solvent B of 95:5 for 2 min, solvent A − solvent B of 80:20 for 1 min, solvent A − solvent B gradient of 80:20 to 50:50 for 2 min, and solvent A − solvent B of 20:80 for 2 min, where solvent A was Milli-Q water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. The data were processed using a computer program (MassLynx; Waters Corp.).

2.5. Biochemical studies

The molecular mass of the enzymes was determined using gel filtration and matrix-assisted laser desorption ionization − time-of-flight mass spectrometry (MALDI–TOF MS). Gel filtration was performed as described in the Supplemental material. MALDI–TOF MS was performed using Autoflex TOF (Bruker Daltonics Inc.). For sample preparation, the purified enzymes (5 mg ml^{-1}) were desalted using dialysis against Milli-Q water. For the molecular mass determination, we chose 2,5-dihydroxybenzoic acid as the MALDI matrix. Effects of pH on hydrolytic activity and the pH stabilities of the enzymes were tested as described in Supplemental materials. Thermostability was tested by incubating 100 μl of a sample (1 mg ml^{-1} protein) for 30 min at temperatures of 30–70 °C. Residual activity was measured using D-Phe-pNA at 25 °C. Fluorescence emission spectra of the enzymes treated with respective temperature for 30 min were recorded using an F4500 fluorescence spectrophotometer (Hitachi, Ltd.). The excitation wavelength was 284 nm, and emission spectra were recorded between 300 and 400 nm in cuvettes with 1 cm path length. The kinetic parameters were determined at 25 °C in a mixture containing 0.1 ml of an enzyme solution (0.1 mg ml^{-1}), 0.1 ml of a 0.1–10 mM D-Phe-pNA solution, and 0.8 ml of 200 mM Tris–malate (pH 6.5). Inhibition of the activity by penicillin G was determined by treating each enzyme with 0.1–10 mM penicillin G at pH 6.5 and 25 °C for 5 min. Residual activity was measured using D-Phe-pNA at pH 6.5 and 25 °C.

2.6. Peptide bond formation by aminolysis reaction

Peptide synthesis by aminolysis reaction was performed as follows: 2 μl of aminoacyl esters dissolved in DMSO (0.5 M) was added to 46 μl of 0.25 M Tris–HCl (pH 9.0). The reaction was initiated by adding 2 μl of 0.2 mg ml^{-1} purified enzyme. The reaction was then continued at 25 °C for an appropriate time (5 min–72 h). The reaction was terminated by adding 0.05 ml of 3% formic acid. The molecular mass of peptides synthesized by the enzymes was determined using ESI–TOF MS, for which the reaction mixture was diluted with 200 or 4000-fold volume of 0.1% formic acid. After the solution was filtered, 5 μl of each sample was analyzed using an ESI–TOF MS system (LCT Premier XE; Waters Corp.). Synthesized D-Phe–D-Phe, D-Phe–D-Phe–D-Phe ($(D\text{-Phe})_3$), hydrolysate D-Phe, and substrate D-Phe-OMe were quantified using UPLC − ESI–TOF MS equipped with a C18 reverse-phase system. The reaction mixture was diluted with 4000-fold volume of 0.1% formic acid and filtered; then 5 μl of each sample was subjected to chromatography. Each sample was eluted and analyzed as described in Section 2.4. For quantifying the synthesized $(D\text{-Phe})_3$, a standard curve for $(L\text{-Phe})_3$ was used instead of a standard for $(D\text{-Phe})_3$.

2.7. Analysis of length of peptide synthesized from D-Tyr-OMe

A precipitate produced in the 3 h reaction using D-Tyr-OMe was collected by centrifugation, washed twice with distilled water, then dissolved in 100 μl of 50% acetic acid containing 0.1% formic acid. The obtained samples were analyzed using Autoflex TOF (Bruker Daltonics Inc.). For the molecular mass determination, we chose 2,5-dihydroxybenzoic acid as the MALDI matrix.

2.8. Specificity for acyl acceptor

Specificity for acyl acceptor in the aminolysis reaction was evaluated as follows: 2 μl of aminoacyl esters dissolved in DMSO (0.5 M) was added onto 46 μl of 0.25 M Tris–HCl (pH 9.0) containing 20 mM acetylated (Ac-) D-Phe-OMe. The reaction was initiated by adding 2 μl of 0.2 mg ml^{-1} purified enzyme. The reaction was then continued at 25 °C for 1 h. The reaction was terminated by adding 0.05 ml of 3% formic acid. The molecular mass