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Enzymatic dehydrogenation of cyclo(L-Phe–L-Leu) to a bioactive derivative, albonoursin [1]

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Abstract

The cell-free extract of *Streptomyces* sp. KO-2388, an albonoursin-producing strain, was found to catalyze the conversion of cyclo(L-Phe–L-Leu) to albonoursin. The conversion activity was simply determined by measuring the increase in ultraviolet (UV) absorption of the reaction mixture at 317 nm, λ_{max} (ε 25,400) of albonoursin, where cyclo(L-Phe–L-Leu) had no absorption. The optimum pH and temperature of this bioconversion using the cell-free extract were determined to be pH 8.0–9.5 and 60°C, respectively. Under the optimum conditions, 620 mg/l of albonoursin was obtained with a conversion ratio of 62% after 24 h incubation.

Keywords: Bioconversion; Dehydrodiketopiprazines; Cyclic dipeptide; Dioxopiperazine; Piprazinedione

1. Introduction

DKPs (2,5-diketopiperazines, 2,5-dioxopiperazines, cyclic dipeptides) and their derivatives, e.g., dehydrogenated DKPs, are widely distributed in nature as secondary metabolites and some of them have unique bioactivities [1,2]. Albonoursin, one of the dehydrogenated diketopiperazines, has been found to have antibacterial and antitumor activities by Fukushima et al. [3], and an inhibitory activity toward pronuclear fusion of sea urchin eggs by Kobayashi et al. [4].

Organic synthesis of albonoursin and several alkylidene derivatives of 2,5-piperazinedione involved several reactions of not so high yield [5–8]. We found that an albonoursin-producing actinomycete produced a large amount of albonoursin by the addition of cyclo(L-Phe–L-Leu) (CFL), prepared from a dipeptide L-Phe–L-Leu, in the course of fermentation [9], and further that the resting cells of the strain catalyzed the conversion of CFL to albonoursin [10] (Fig. 1). Like fungi, the filaments grown makes it difficult to prepare a high density of the resting cells of actinomycetes. Therefore, soluble enzyme preparation such as a cell-free extract, rather than the resting cells, are thought to be suited for the conversion.

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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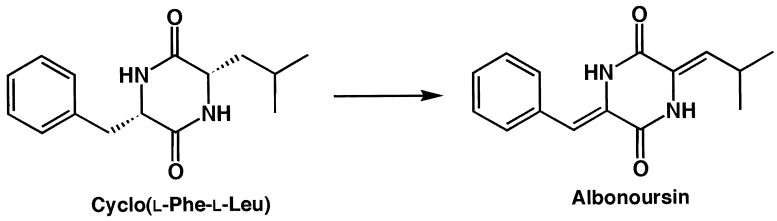

Fig. 1. Scheme of bioconversion reaction.

In the present study, we examine the ability of a cell-free extract prepared from the albonoursin-producing actinomycete to convert CFL to albonoursin and optimize the reaction conditions for the enzymatic conversion.

2. Experimental

2.1. Materials

^{1}H-NMR spectra were recorded with a Varian VXR-500 instrument. Ultraviolet (UV) and mass spectrometry (MS) spectra were obtained with Shimadzu UV-3000 and JEOL SX-102A equipments, respectively. Optical rotation was obtained with a Jasco DIP-360 polarimeter. CFL was prepared from the L-Phe–L-Leu (SIGMA) by the method of Kopple and Ghazaarian [11].

2.1.1. Cyclo(L-Phe–L-Leu)

$[\alpha]_D^{23} + 38.0°$ (c 0.1, CH_3COOH). EIMS m/z (rel. int.): 260 (M^+, 26.0), 204 (39.0), 169 (24.7), 141 (34.2), 113 (25.3), 91 (100.0). IR υ_{max} (KBr) cm^{-1}: 3300, 3191, 1657, 1494. UV λ_{max} (MeOH) nm (ε): 247 (110), 252 (280), 256 (338), 263 (260). NMR δ_H (DMSO-d_6): 0.12 (1H, m), 0.58 (3H, d, $J = 6.4$ Hz), 0.63 (3H, d, $J = 6.7$ Hz), 0.75 (1H, m), 1.42 (1H, m), 2.83 (1H, dd, $J = 4.9$, 13.4 Hz), 3.13 (1H, dd, $J = 3.7$, 13.4 Hz), 3.47 (1H, m), 4.16 (1H, ddd, $J = 1.5$, 3.7, 4.9 Hz, 7.13 (2H, d, $J = 7.9$ Hz), 7.22 (1H, t, $J = 7.6$ Hz), 7.27 (2H, dd, $J = 7.6$, 7.9 Hz), 8.07 (1H, br.s), 8.09 (1H, br.s).

2.2. Cultivation

The cells of *Streptomyces* sp. KO-2388 were found to have the highest conversion activity of CFL to albonoursin after 2 days of cultivation [10]. Thus, we used the 2-day cultured cells for the following experiments. Strain KO-2388 was cultivated on agar slants (18 mm dia. tube) of Bennett's medium (1 g/l of yeast extract (Nacalai Tesque) 1 g/l of beef extract (DIFCO), 2 g/l of NZ Amine type A (Humko Sheffield Chemical), 10 g/l of glucose, and 15 g/l of agar at pH 7.3) at 28°C for 14 days. A spore and aerial mycelia suspension was prepared by adding 10 ml of sterilized water containing two drops of Triton X-100. A total of 40 μl of this suspension was used to inoculate 40 ml of the medium KP (15.0 g/l of glucose, 10.0 g/l of glycerol, 10.0 g/l of Polypepton, 10.0 g/l of beef extract and 4.0 g/l of $CaCO_3$ at pH 7.3) in 200 ml Erlenmeyer flasks. After cultivating on a rotary shaker (180 rpm) at 28°C for 48 h, the cells were harvested by centrifugation, washed with 0.85% NaCl, suspended in a buffer to make a thick slurry.

2.3. Enzyme assay

The assay for albonoursin-producing activity was carried out at 37°C by measuring an increase in absorbance at 317 nm, λ_{max} of albonoursin. The reaction mixture contained 0.29 μmol of CFL (in 50 μl of DMSO), a buffer, and an enzyme solution in a total volume of 0.5 ml.

One unit of the conversion activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of albonoursin per minute under the standard conditions.

Protein was determined by the Bio-Rad assay method based on Coomassie brilliant blue dye binding procedure [12] in which bovine serum albumin served as the standard protein. Specific activity is expressed as unit activity per mg of protein.

2.4. Determination of albonoursin

A total of 500 μl of the reaction mixture was extracted with ethyl acetate. The ethyl acetate fraction was concentrated and dissolved in 250 μl of MeOH. Around 1 μl of the MeOH solution was spotted on a silica gel plate (E. Merck, Kieselgel 60 F_{254}, Art. 5554) and developed with benzene–ethyl acetate (6:4). Albonoursin (Rf = 0.67) was determined under UV light (317 nm) by a Shimadzu flying-spot scanner CS-9000 in a range of 11.4–114 ng.

3. Results and discussion

3.1. Detection of activity converting CFL to albonoursin

The harvested cells of *Streptomyces* sp. KO-2388 was disrupted with an ultrasonic oscillator (150 W with cooling for 2 min, KUBOTA Insonator 201 M). The cell-free extract was obtained by centrifugation and the remaining cell debris was suspended with the Britton and Robinson buffer (pH 10.0). The resting-cells, the cell-free extract, and the cell debris suspension were tested for the conversion activity. The conversion activity was measured under the optimum conditions for the resting cell reaction, using the Britton and Robinson buffer (pH 10.0) [11]. The reaction mixture contained 2.9 μmol of CFL (500 μl of DMSO), 63 μmol of the Britton and Robinson buffer (pH 10.0), and each enzyme preparation described above in a total volume of 5.0 ml. The reaction was carried out at 50°C for 2 h with shaking (160 strokes/min). After the reaction, albonoursin in

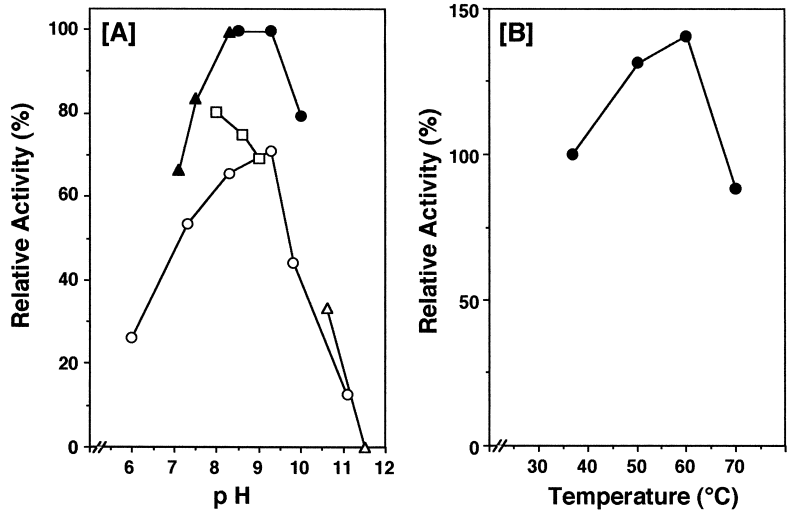

Fig. 2. Optimum pH and temperature of the conversion reaction. [A] Effect of pH on the reaction. The reaction was performed under the standard conditions using the following buffers: sodium phosphate buffer (▲), boric acid/NaOH buffer (●), $NaHCO_3$/NaOH buffer (△), Bicine/NaOH buffer (□), Britton and Robinson buffer (○). [B] Effect of temperature on the reaction. The reaction was performed under the standard conditions at various temperatures.

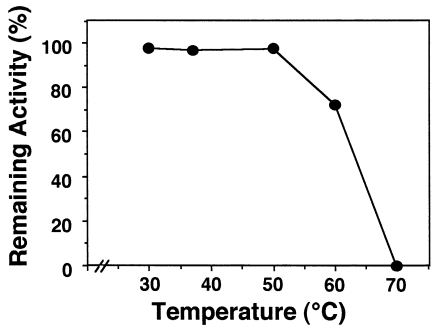

Fig. 3. Stability of the enzyme activity. After the incubation of the cell-free extract at various temperature for 3 h, the remaining converting activity was measured under the standard conditions.

EtOAc extract of the reaction mixture was determined by the method described in Section 2.4.

The cell-free extract exhibited 60% of the conversion activity shown by the resting-cells, while the cell debris suspension had little activity. These results indicated that the enzyme system converting CFL to albonoursin was localized in cytosol of the cells and was obtained as a stable cell-free extract. This cell-free extract was used throughout the following experiments.

Determination of the enzyme activity essential for the effective production of albonoursin was conducted by measuring the initial rate of albonoursin formation (cf. Section 2.3).

3.2. Optimum pH and temperature for the conversion activity

The effect of pH on the conversion activity of the cell-free extract was examined (Fig. 2A). The cell-free extract exhibited the highest activity at pH 8.0–9.5, while the resting-cells exhibited it at pH 10.0 [10]. Based on these data, the standard enzyme assay in the following experiments was performed in 10 mM sodium phosphate buffer (pH 8.0) at 37°C. The extract exhibited the specific activity of 3.5×10^{-3} units/mg under the standard conditions. The difference in optimum pH between the cell-free extract and the resting cells is thought to be due to the difference in permeability of the substrate or product. Ionic strength of sodium phosphate buffer did not affect the activity.

The effect of temperature on the conversion activity of the cell-free extract was also examined similarly at various temperature (Fig. 2B). The cell-free extract exhibited the highest activity at 60°C, while the resting-cells exhibited it at 50°C [10].

3.3. Heat stability of the converting enzyme

After the incubation of the cell-free extract at various temperature for 3 h, the remaining converting activity was measured under the standard conditions. No loss of activity was observed when the extract was incubated at temperatures of up to 50°C (Fig. 3). Therefore, the conversion reaction from CFL to albonoursin by the cell-free extract in the following experiments was carried out at 50°C.

3.4. Effect of substrate concentration on the conversion

The conversion by the cell-free extracts was examined with different substrate concentrations (Fig. 4). The reaction mixture contained CFL

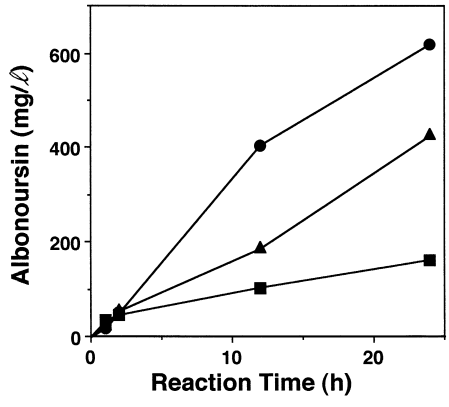

Fig. 4. Effect of the substrate concentration on the conversion reaction. The reaction was carried out at 50°C for 24 h. Each reaction mixture (5.0 ml) contained 450 μmol of sodium phosphate buffer (pH 8.0), the cell-free extract (0.06 units) and 0.5 ml solution of CFL (5 mg ●, 2.5 mg ▲, 0.75 mg ■) in DMSO.

(in 500 μl of DMSO), 45 μmol of sodium phosphate buffer (pH 8.0), and the cell-free extract (0.06 units) in a total volume of 5.0 ml. Taking into account the enzyme stability, the reaction was performed at 50°C, lower than the optimum temperature. During the reaction, aliquots of the reaction mixture were taken and the albonoursin content was determined by the method described in Section 2.4. In the mixture containing 0.75 mg of CFL (150 mg/l), albonoursin was produced in 100% yield after 24 h incubation. From 5 mg of CFL, 3.1 mg of albonoursin was obtained after 24 h with a conversion ratio of 62%.

3.5. Purification and identification of conversion product

A reaction mixture containing 0.27 units of enzyme, 25 mg of CFL (in 5.0 ml of DMSO) and 350 μmol of sodium phosphate buffer (pH 8.0) in a total volume of 40 ml was incubated at 50°C with shaking (160 strokes/min). After 24 h, precipitates in the mixture were collected by centrifugation and were found to contain albonoursin by a TLC analysis. The precipitates (14 mg) were subjected to chromatography on silica gel (Wakogel C-300, ϕ 0.4×100 cm) with elution by benzene–ethyl acetate (9:1). The fractions containing compound **1** of the same Rf value (0.57, developing solvent: benzene–ethyl acetate (6:4)) as albonoursin on TLC were combined (3.8 mg). Compound **1** was identified as 3-benzylidene-6-isobutylidene-2,5-piperazinedione by direct comparison with the product isolated from the culture broth of *Streptomyces* sp. KO-2388. Furthermore, the geometry of compound **1** was found to be (3*Z*, 6*Z*) by NOESY analysis, indicating that compound **1** had the same stereochemistry as albonoursin (Fig. 5).

*3.5.1. Compound **1***

HREIMS m/z (M^+):calcd. for $C_{15}H_{16}N_2O_2$:256.1212, Found: 256.1250. UV λ_{max} (MeOH) nm (ε): 235 (9000), 317 (25,400).

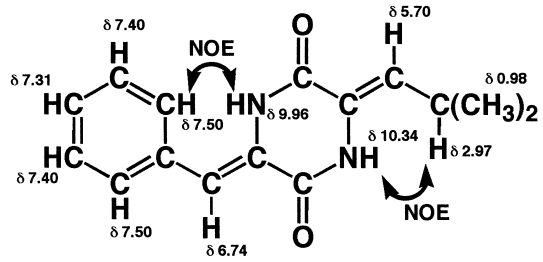

Fig. 5. Structure of albonoursin, 3-(*Z*)-benzylidene-6-(*Z*)-isobutylidene-2,5-piperazinedione.

NMR δ_H (DMSO-d_6): 0.98 (6H, d, $J = 6.7$ Hz), 2.97 (1H, dsep, $J = 6.7$, 10.4 Hz), 5.70 (1H, d, $J = 10.4$ Hz), 6.74 (1H, s), 7.31 (1H, t, $J = 7.6$ Hz), 7.40 (2H, t, $J = 7.6$ Hz), 7.50 (2H, d, $J = 7.6$ Hz), 9.96 (1H, br.s), 10.34 (1H, br.s).

Our preliminary experiments indicated that the cell-free extract catalyzed the conversion of several diketopiperazines to their dehydroderivatives. The fact that albonoursin, a dehydroderivative of CFL, has a pronounced inhibitory activity toward pronuclear fusion of sea urchin eggs while CFL itself has not, suggested that other dehydrogenated diketopiperazines are potential resources of unique bioactivity. This effective enzymatic conversion system of CFL to albonoursin were promising for mass production of these dehydrogenated diketopiperazines.

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