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Effective Production of Dehydro Cyclic Dipeptide Albonoursin Exhibiting Pronuclear Fusion Inhibitory Activity

II. Biosynthetic and Bioconversion Studies

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Albonoursin production was greatly enhanced when cyclo (L-Leu-L-Phe) (CFL), a tetrahydro derivative of albonoursin, was added to the 2-day culture of an albonoursin-producing actinomycete, *Streptomyces albulus* KO-23. The increase in albonoursin production paralleled the amount of CFL added. Furthermore, the resting cells of the strain catalyzed the bioconversion of CFL to albonoursin. The optimum pH and temperature for the conversion were found to be pH 10.0 and 50°C. The feeding experiments and the resting-cell reactions revealed that albonoursin is biosynthesized by dehydrogenation of CFL in the actinomycete. This is the first report for a dehydrogenation of amino acid residues at the α,β-positions in cyclic dipeptides.

Actinomycete strain KO-23[1] produces albonoursin[2], a dehydro derivative of a 2,5-diketopiperazine (DKP). Albonoursin exhibits pronounced inhibitory activity toward pronuclear fusion of sea urchin eggs[2]. Strain KO-23 was identified as *Streptomyces albulus* in the preceding paper[3]. The optimum conditions for the fermentation of albonoursin were also determined[3]. Under these conditions, the biosynthetic enzymes for albonoursin are highly expressed. In this paper, we report biosynthetic and bioconversion studies on albonoursin.

Materials and Methods

Materials

Cyclo (L-Leu-L-Phe), CFL, was prepared from L-Leu-L-Phe (SIGMA) by the method of KOPPLE and GHAZAARIAN[4]. ^{1}H-NMR spectra were recorded with a Varian VXR-500 instrument. UV and MS spectra were obtained with Shimadzu UV-3000 and JEOL SX-102A instruments, respectively. The product is determined to be sterically pure by comparing its optical rotation obtained with a Jasco DIP-360 polarimeter with that of CFL reported by NITECKI *et al.*[5]

Cyclo (L-Leu-L-Phe), CFL. $[\alpha]_D^{23}$ +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} (Methanol) 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).

Cultivation of an Albonoursin-producing Strain

Strain KO-23 was cultivated at 28°C for 14 days on agar slants (10 ml in 18-mm dia. tubes) of Bennett's medium containing per liter 1 g of yeast extract (Nacalai Tesque), 1 g of beef extract (DIFCO), 2 g of NZ Amine type A (Humko Sheffield Chemical), 10 g of glucose, and 20 g of

agar at pH 7.3. A spore and aerial mycelium suspension was prepared by adding 10 ml of sterilized water containing two drops of Triton X-100. Forty μl of the spore suspension were used to inoculate a 200-ml Erlenmeyer flask containing 40 ml of medium KP containing per liter of 15.0 g of glucose, 10.0 g of glycerol, 10.0 g of Polypepton, 10.0 g of beef extract, and 4.0 g of $CaCO_3$ (pH 7.3). Cultivation was carried out at 28°C on a rotary shaker (180 rpm).

Fed-batch Cultivation

After 2-day cultivation described above, CFL solution in DMSO was fed to the culture of strain KO-23, keeping the DMSO final concentration below 10%. DMSO did not affect the growth of strain KO-23 at a concentration of 10%.

Resting Cell Reaction

Resting cells were prepared by centrifugation of a culture produced on a rotary shaker (180 rpm) in 40 ml of the medium KP at 28°C for 48 hours, followed by washing the cells twice with 0.85% NaCl and resuspending them in 8 ml of the physiological saline. The final concentration of resting cells prepared by this procedure was about 250 mg of wet cells per ml. The reaction mixture consisted of 4.5 ml of the suspension of the resting cells and 0.5 ml of CFL solution in DMSO. After appropriate times of incubation at 160 strokes/minute, the reaction was terminated by adding 1-ml aliquots of the reaction mixture to the 5 ml of methanol. After removing the cells by centrifugation, the albonoursin content was determined by HPLC.

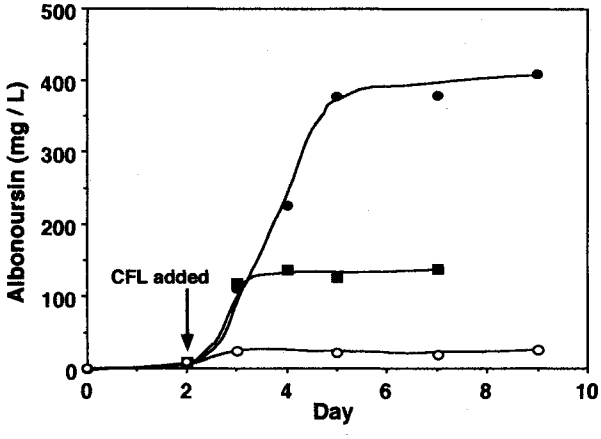
Determination of Albonoursin

Culture broth obtained by fed-batch culture was separated into the supernatant and the cells by centrifugation. The supernatant was extracted with ethyl acetate and the cells with methanol - acetone (1 : 1). The ethyl acetate extract of the supernatant and the methanol - acetone extract of the cells were then subjected to TLC or HPLC analysis.

TLC analysis: One μl of the methanol solution of albonoursin-containing sample 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.

HPLC-analysis: The albonoursin content was determined by HPLC on an Inertsil ODS-3 column (i.d. 4.6×250 mm, GL Sciences) with UV detection at 317 nm. Albonoursin eluted in 19.0 minutes with 60% methanol at a flow rate of 1.0 ml/minute.

Fig. 1. Fed-batch culture of the albonoursin-producing strain.



Cyclo (Leu-Phe) in DMSO was added to the 2-day culture at final concentrations of 150 (■) and 500 (●) mg per liter. Open circles indicate data for albonoursin production without CFL addition.

Results

Fed-batch Culture

Since cyclo (L-Leu-L-Phe), a tetrahydro derivative of albonoursin, was a possible biosynthetic precursor of albonoursin, we carried out experiments feeding CFL to cultures of strain KO-23. In the preceding paper[3], we reported that albonoursin production increased after 2-day cultivation, indicating that a biosynthetic enzyme system for albonoursin was also highly expressed after two days. When CFL in DMSO was added to the culture after 2-day cultivation, albonoursin accumulation was enhanced with the enhancement proportional to the dose of CFL, as shown in Fig. 1. This result indicated that albonoursin is biosynthesized from CFL by the strain (Fig. 2).

Resting Cell Reaction

The ability of the resting cells to catalyze the bioconversion of CFL to albonoursin was tested. The resting cells suspended in 0.85% NaCl were found to catalyze the bioconversion. In the presence of 150 mg CFL per liter in the reaction mixture, albonoursin production increased with incubation time at 37°C and went up to over 130 mg/L after

Fig. 2. Bioconversion of CFL to albonoursin catalyzed by *Streptomyces albulus* KO-23.

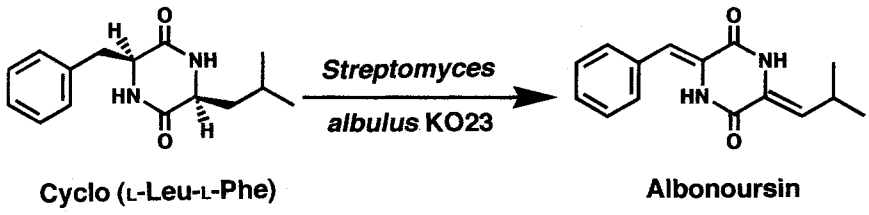
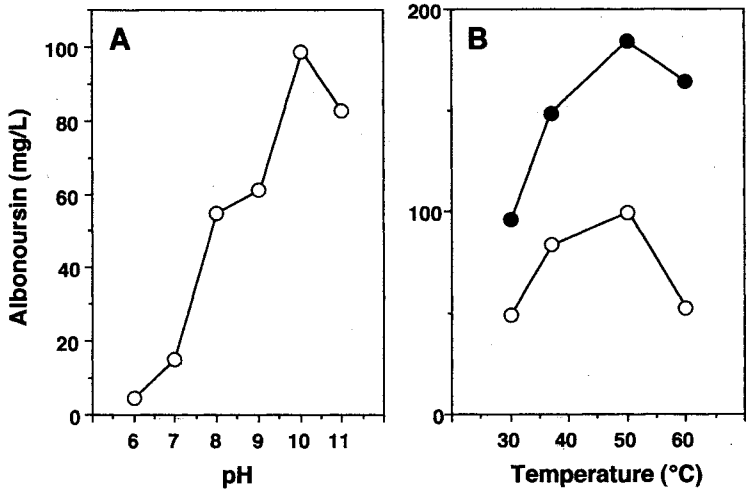


Fig. 3. Optimum pH and temperature of bioconversion of CFL to albonoursin catalyzed by resting cells of *Streptomyces albulus* KO-23.



A: Albonoursin production versus pH,
B: Albonoursin production at different temperatures for 1 (○) and 2 hours (●) incubation

12-hours incubation. However the yield was not reproducible because the pH of the mixture varied throughout the reaction, and the variation affected the albonoursin production. Therefore, we tried to determine an optimum pH for the bioconversion.

Optimum pH

We selected a Britton-Robinson buffer system[6] for determination of the optimum pH for this bioconversion because a wide range of pH is available in this buffer. As shown in Fig. 3A, albonoursin production from CFL by the resting KO-23 cells was found to be high at alkaline pH with a maximum at pH 10.0.

Optimum Temperature

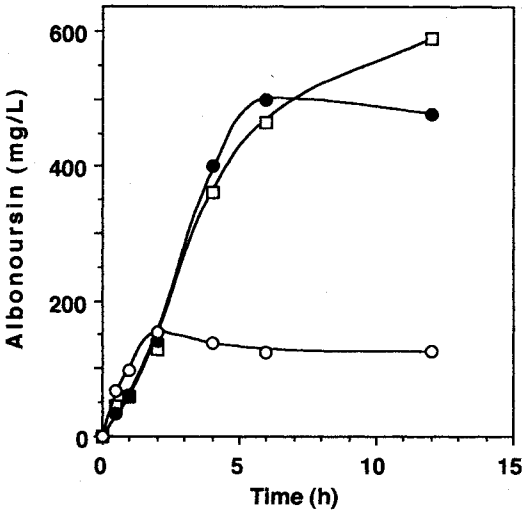
We determined the optimum temperature for this bioconversion at the optimum pH of the reaction (pH 10.0). The highest production was attained at 50°C for both 1 and 2 hours incubations (Fig. 3B).

Effect of Substrate Concentration

Production of albonoursin increased with increasing substrate concentration at 50°C and pH 10.0 (Fig. 4). At CFL concentrations of 150 and 500 mg per liter, the substrate was completely converted to albonoursin. In the presence of 1000 mg per liter of CFL, more than 600 mg of albonoursin per liter was produced after 12-hours incubation, and the conversion was still in progress at that time.

The product from CFL converted by the resting cells was

Fig. 4. Albonourin production by resting cells.



The reaction was carried out at CFL concentrations of 150 (○), 500 (●), and 1000 (□) mg per liter at 50°C and pH 10.0.

isolated and identified as 3-benzylidene-6-isobutylidene-2,5-piperazinedione by direct comparison of its spectral data with those of the compound isolated from the culture broth of *Streptomyces albulus* KO23[2] or prepared from CFL converted by the cell-free extract[7]. Furthermore, its geometry was found to be (3*Z*, 6*Z*) by NOESY analysis, indicating that the compound had the same stereochemistry as albonoursin (Fig. 2).

Characterization of Resting Cells

Strain KO-23 attained its stationary phase after 2-day cultivation under the conditions described in Materials and Methods. The specific activity for the conversion, which was represented by the amounts of albonoursin produced per mg of wet cells, remained high from 1- through 5-day cultivation. These results indicated that the resting cells obtained from 2-day cultivation were the best as a catalyst for this bioconversion.

The stability of the enzyme system in the resting cells was tested at 5 and −80°C. The cells were gradually inactivated under both conditions, but over 80% of the activity remained in the cells stored at both temperatures for 5 days.

Discussion

In this paper, we described using an albonoursin-producing actinomycete for the bioconversion of CFL to albonoursin, which is an example of the biosynthesis of dehydroamino acid-containing peptides. We have already revealed that the cell-free extract of this actinomycete catalyzed the bioconversion[7] and that the conversion required phenazine methosulfate as a cofactor[8] and proceeded in the absence of O_2 (data not shown). These results strongly suggested that the reaction is a dehydrogenation, not a dehydration or an O_2-dependent oxidation.

Few studies have dealt with the biosynthetic pathway for dehydroamino acid-containing peptides, although microorganisms or plants have been known to produce a variety of these peptides. However, the biosynthetic pathway has been studied in lantibiotics[9], bacteriocins produced by lactic acid bacteria. Lanthionine residues in lantibiotics are known to be biosynthesized by condensation of dehydroamino acid and cysteine residues, constituting a post-translational modification of peptide chains. Dehydroamino acids involved in the posttranslational modification of lantibiotics are dehydroalanine from serine and dehydrobutyrine from threonine by a dehydration. Therefore, the reactions involved in lantibiotic biosynthesis are found to be different from the bioconversion reaction described in this paper. A second example is tryptophan side chain oxidase reported by TAKAI and HAYAISHI[10]. This enzyme catalyzes the formation of dehydrotryptophan, but requires O_2 for the reaction. Thus, this enzyme is also thought to be distinct from the albonoursin-forming enzyme. These facts suggest that the enzyme system catalyzing the bioconversion of CFL to albonoursin is the first example for the dehydrogenation of amino acid residues in peptides.

High productivity of albonoursin was attained by this bioconversion system using strain KO-23 (Fig. 2). Furthermore, we found in our preliminary experiments that this system was capable of synthesizing other dehydro cyclic dipeptides from the corresponding cyclic dipeptides. Therefore, this system would be promising for the production of novel bioactive dehydro cyclic dipeptides.

Acknowledgments

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