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

I. Taxonomy and Fermentation

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Strain KO-23, an actinomycete producing albonoursin as well as streptopyrone, was identified as *Streptomyces albulus* by morphological and biochemical studies. Fermentation conditions for albonoursin, a dehydro cyclic dipeptide exhibiting a pronounced inhibitory activity toward pronuclear fusion of sea urchin eggs, were optimized. Under the optimum conditions, the actinomycete produced 16 mg/liter of albonoursin, 30 times higher than that in the original culture. The cells cultivated under these conditions highly express biosynthetic enzymes for albonoursin, and thus are available for biosynthetic studies of dehydro cyclic peptides.

2,5-Diketopiperazines (DKPs, 2,5-dioxopiperazines, cyclic dipeptides), are widely distributed in nature as secondary metabolites and are known to exhibit a variety of bioactivity[1,2]. Some of them are present in dehydrogenated form containing dehydroamino acid residues. We found that *Streptomyces* sp. KO-23, a novel γ-pyrone-producing strain[3], also produced albonoursin, the tetradehydro-derivative of cyclo (Leu-Phe)[4] which exhibits an inhibitory activity against pronuclear fusion of sea urchin eggs[5]. Albonoursin is known to have antibacterial and antitumor activities[6]. In spite of many reports on structural elucidation and bioactivities of DKPs and their dehydro-derivatives, little is known about the biosynthetic relationship between DKPs and their dehydro-derivatives.

In this paper, we report the taxonomy of the strain and the optimum culture conditions for albonoursin fermentation. The cells cultivated under these conditions have highly expressed biosynthetic enzymes for albonoursin. The biosynthetic and bioconversion studies of albonoursin will be separately reported[7].

Materials and Methods

Producing Microorganism and Taxonomy

To examine the morphological and cultural properties, strain KO-23 was cultured by the methods described in the International Streptomyces Project[8,9] and observed under light and scanning electron microscopes. The culture was examined after incubation at 27°C for 14 days. The physiological properties including carbon utilization were examined as described by PRIDHAM and GOTTLIB[10]. The stereochemistry of diaminopimelic acid formed by hydrolyzing the whole-cell was elucidated by the method of STANECK and ROBERTS[11].

Fermentation

Strain KO-23 was cultivated at 28°C for 14 days on agar slants (18-mm dia. tube) of Bennett's medium (10 ml) 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

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was prepared by adding 10 ml of sterilized water containing two drops of Triton X-100. This suspension was used to inoculate the various media described below. Forty μl and 150 μl of the spore suspension were used to inoculate 40 ml of the medium in 200-ml Erlenmeyer flasks and 150 ml in 500-ml Sakaguchi flasks, respectively. Cultivation of the cells was carried out at 28°C in Erlenmeyer flasks on a rotary shaker (180 rpm) or in Sakaguchi flasks on a reciprocal shaker (120 strokes/minute).

Various Media Screened

Medium PSM, per liter: 200 g of peeled potato, 20 g of sucrose, and 2 g of malt extract (DIFCO); medium KA, per liter: 35.0 g of malt extract, 30.0 g of corn starch, 15.0 g of corn steep liquor (Ajinomoto), 15.0 g of Pharmamedia (Procter & Gamble Oilseed Products), 5.0 g of Sungrain (Sungrain Corporation), and 2.0 g of $CaCO_3$ (pH 7.3); medium KF, per liter: 10.0 g of glucose, 5.0 g of glycerol, 10.0 g of corn steep liquor, 10.0 g of soybean meal (Nisshin Oil Mills), 5.0 g of dry yeast (Asahi Breweries), 5.0 g of NaCl, and 2.0 g of $CaCO_3$ (pH 5.7); medium KG, per liter: 10.0 g of starch, 5.0 g of Polypepton (Nihon Pharmaceutical), 10.0 g of cane molasses (Ajinomoto), 10.0 g of beef extract (DIFCO), and 2.0 g of $CaCO_3$ (pH 6.9); medium KH, per liter: 15.0 g of glucose, 10.0 g of glycerol, 15.0 g of soybean meal, 5.0 g of dry yeast, 5.0 g of NaCl, 5.0 g of $(NH_4)_2SO_4$, 10.0 g of starch, 10.0 g of Polypepton, 20.0 g of cane molasses, 10.0 g of beef extract, and 4.0 g of $CaCO_3$ (pH 7.2); medium KK, per liter: 25.0 g of starch, 15.0 g of soybean meal, 2.0 g of dry yeast, and 4.0 g of $CaCO_3$ (pH 7.0); medium KN, per liter: 15.0 g of soybean meal, 2.0 g of $(NH_4)_2SO_4$, 2.0 g of dry yeast, 25.0 g of starch, 5.0 g of NaCl, and 4.0 g of $CaCO_3$ (pH 7.5); medium KN2, per liter: 25.0 g of starch, 30.0 g of glucose, 10.0 g of Polypepton 20.0 g of corn steep liquor, and 2.0 g of NaCl (pH 7.0); medium KP, per liter: 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); medium KQ, per liter: 20 g of glycerol, 5 g of casein, 10 g of cane molasses, 1.0 g of Polypepton, and 4 g of $CaCO_3$ (pH 7.2); medium KCM, per liter: 10.0 g of glucose, 5.0 g of glycerol, 3.0 g of corn steep liquor, 3.0 g of beef extract, 3.0 g of malt extract, 3.0 g of yeast extract, 2.0 g of $CaCO_3$, and 0.01 g of thiamine (pH 7.0); medium KPY, per liter: 10.0 g of starch, 10.0 g of glycerol, 5.0 g of glucose, 5.0 g of beef extract, 3.0 g of Polypepton, 2.0 g of yeast extract, 1.0 g of casein, 2.0 g of $CaCO_3$, and 0.01 g of thiamine (pH 7.0); medium K2, per liter: 40.0 g of glutinous starch syrup, 20.0 g of soybean meal, 10.0 g of Pharmamedia, 5.0 g of Sungrain, 3.0 g of soybean oil (Wako Pure Chemical Industries), 5.0 g of $CaCO_3$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $CoCl_2 \cdot 6H_2O$, and 0.01 g of $NiCl_3 \cdot 6H_2O$ (pH 7.3); medium KFA, per liter: 20.0 g of corn starch, 5.0 g of glucose, 5 g of soybean oil, 20 g of soybean meal, 5.0 g of Pharmamedia, 1 g of Staminol, and 2.0 g of $CaCO_3$ (pH 7.3).

Preparation of Test Solutions to be Analyzed

After cultivating at 28°C, the culture broth was centrifuged and the supernatant was extracted with ethyl acetate. The ethyl acetate extract obtained was then subjected to a bioassay or an HPLC analysis.

Bioassay for Cytotoxicity[3,12]

Sexually mature sea urchins (*Hemicentrotus pulcherrimus*) were collected during the breeding season (January~March) from the coastal water near Ushimado Marine Laboratory, Faculty of Science, Okayama University. Eggs and sperm were obtained by KCl shedding. Eggs were agitated and left for 3 minutes. The eggs near the surface and the bottom were removed by decantation and suction. This process provided better fertilization and synchronous development of the eggs. The sperm shedded from genital papillae was collected with a glass capillary (dry sperm) and stored in a refrigerator (4°C) until use.

Eggs (*ca.* 4×10^3) were inseminated in a sperm suspension (1 ml, *ca.* 1000 times dilution of the dry sperm). Following fertilization, the fertilization membrane is raised and a hyaline layer is formed. After the hyaline layers were observed with a light microscope, the fertilized eggs (*ca.* 100) were placed in the sea water (1 ml) containing the serially diluted test extracts in a 24-well plastic plate. The cytotoxicity was judged by observing the inhibition of the first cleavage of sea urchin embryos 4 hours after fertilization. One unit of cytotoxicity is defined as the amount equivalent to the minimum inhibitory dose for the first cleavage.

Determination of the Albonoursin Content by HPLC

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 at 5.4 minutes with 80% MeOH at a flow rate of 1.0 ml/minute

Results and Discussion

Taxonomy of the Albonoursin-producing Strain, KO-23

Strain KO-23 produced well-branched vegetative mycelia

Table 1. Cultural characteristics of strain KO-23.

Medium	Growth	Aerial mycerium	Reverse side color	Soluble pigment
Sucrose-nitrate agar	moderate	moderate, gray	gray	none
Sucrose-asparagine agar	moderate	abundant, gray ~ light brown	off-white	none
Glycerol-asparagine agar (ISP No.5)	good	abundant, white ~gray	off-white, light olive	none
Inorganic salt-starch agar	good	abundant, white ~ gray	off-white, light olive	none
Tyrosine agar (ISP No.7)	good	abundant, white	off-white	none
Yeast extract-malt extract agar (ISP No.2)	good	abundant, gray	off-white	none
Oatmeal agar (ISP No. 3)	good	abundant, gray ~ light brown	off-white	none
Bennett's agar	good	abundant, gray	off-white	none
Calcium malate agar	moderate	moderate, white	white	none
Nutrient agar	good	abundant, white ~ gray	off-white	none

Observation after incubation at 27°C for 14 days.

on both synthetic and complex media and did not show fragmentation into coccoid forms or bacillary elements. This strain formed long aerial hyphae which bore spirals of 6 to 9 turns. The spores were ovoid in shape, 0.6~0.8×0.9~1.0 μm in size, and had a spiny surface (Fig. 1). No synnemata, sclerotia or sporangia were observed.

The cultural characteristics of strain KO-23 are shown in Table 1. The vegetative mycelia were white to gray on various media. The aerial mass was white to gray. No soluble pigment was produced on any agar plates. The physiological characteristics and carbohydrate utilizations are shown in Table 2. Permissive temperature ranges for growth of the strain were 10 to 30°C.

Whole-cell hydrolyzates of the strain contained L,L-diaminopimelic acid and glycine, indicating that strain KO-23 has a type I cell wall.

Based on these characteristics, strain KO-23 belongs to the genus *Streptomyces*. A search of the taxonomic data of known *Streptomyces* species in the ISP descriptions by SHIRLING and GOTTLIEB[13~15] led to the identification of strain KO-23 as *Streptomyces albulus*. Strain KO-23 (same as strain MCI 2425) has been deposited in the National Institutes of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan, under

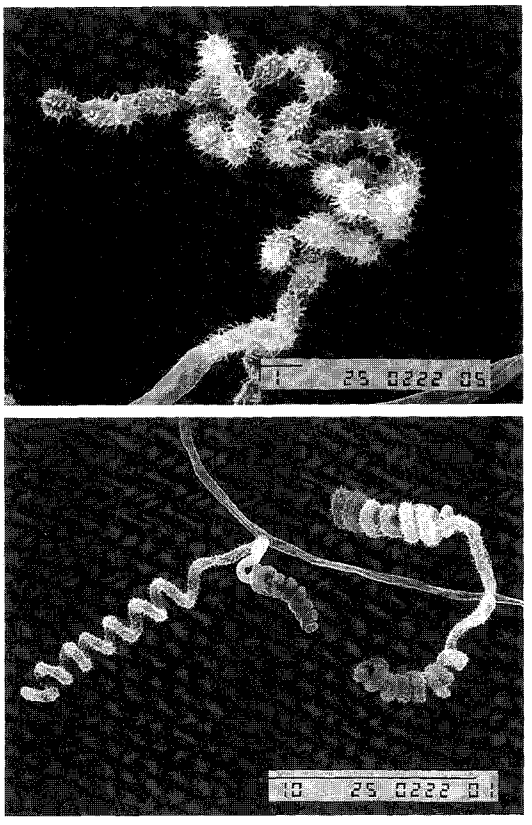
Table 2. Physiological characteristics of strain KO-23.

Condition	Characteristic
Temperature range for growth (°C)	10 - 30
Production of melanoid pigments	-
Starch hydrolysis	+
Geratin liquefaction	+
Milk coagulation	-
Milk peptonization	+
Nitrate reduction	-
Carbohydrate utilization:	
D-Glucose	+
L-Arabinose	-
D-Xylose	-
D-Fructose	+
Sucrose	-
L-Rhamnose	-
Raffinose	-
Inositol	+
D-Mannitol	+

+: positive, -: negative

the accession No. FERM P-10567.

Fig. 1. Scanning electron micrograph of spore chains of *Streptomyces albulus* KO-23 grown for 14 days.



Optimum Culture Conditions for Albonoursin Fermentation

First Screening for Optimum Medium

Production of secondary metabolites in actinomycetes is known to be much affected by the method of shaking, *i.e.*, rotary or reciprocal shaking, and the components of the media. Thus, we examined the production of cytotoxic compounds by strain KO-23 under 30 different culture conditions by combining the two shaking methods and the 15 different culture media described in Materials and Methods. We used 200-ml Erlenmeyer flasks for rotary shaking and 1-liter Sakaguchi flasks for reciprocal shaking.

When strain KO-23 was cultivated in medium PSM, little cytotoxic compounds were produced irrespective of the shaking method. On the other hand, the highest production of cytotoxic compounds was attained under five culture conditions, *i.e.*, medium KP-rotary shaking, medium KF-rotary shaking, medium KK- reciprocal shaking, medium KF-reciprocal shaking or medium KQ- reciprocal shaking (Fig. 2). The difference in production of cytotoxic compounds in the same medium between the two methods of shaking indicated that aeration rate or shear stress greatly affected the production.

Fig. 2. Production of cytotoxic compounds by strain KO-23.

The ▨ and ▩ bars indicate the cytotoxicity units of EtOAc extracts from culture supernatant obtained by rotary and reciprocal shakings, respectively.

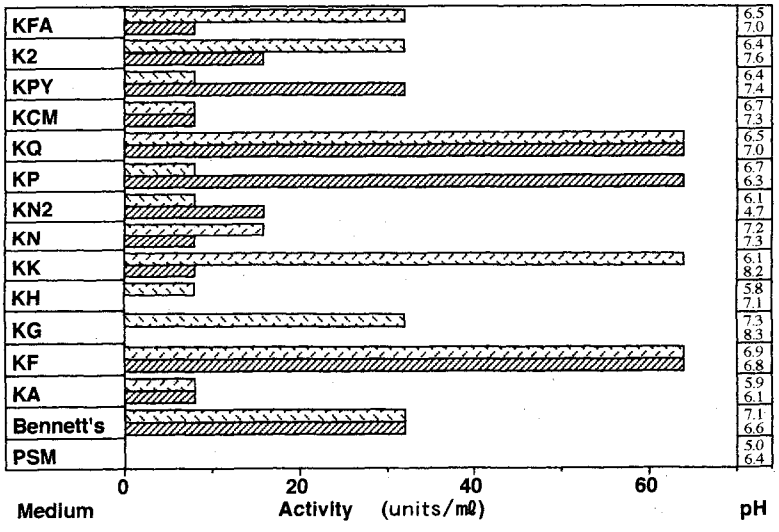
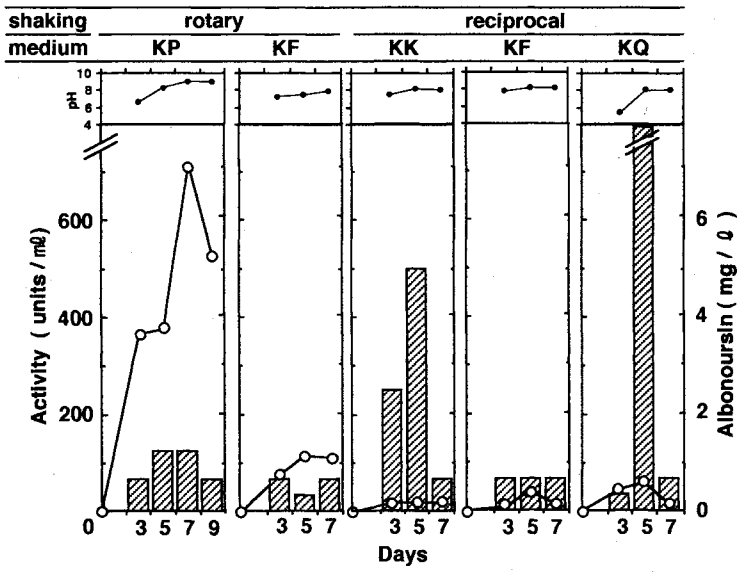


Fig. 3. Production of albonoursin by several different culture conditions.

The ▨ bar and the open circle (○) indicate the cytotoxicity units and amounts of albonoursin respectively.



Second Screening for Optimum Medium

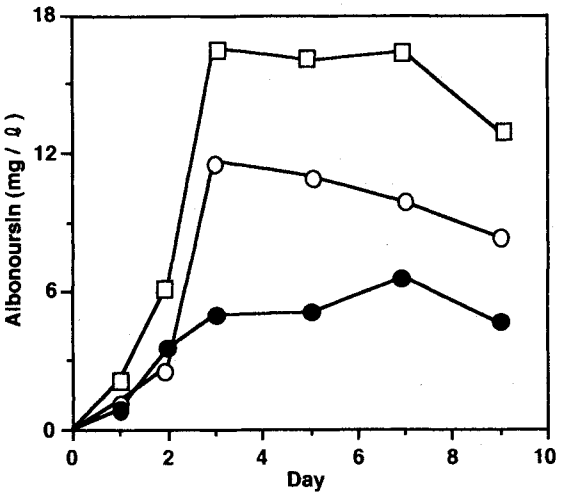
Since strain KO-23 was expected to produce considerable amounts of albonoursin under the promising culture conditions described above, we carried out a detail examination of albonoursin production. The culture in rotary-shaken medium KP was found to be best for albonoursin production by strain KO-23 (Fig. 3). Fig. 3 shows that the strain produced considerable amounts of other cytotoxic compounds in reciprocally shaken medium KK or medium KQ. Since little streptopyrone was detected in these extracts by TLC analysis, strain KO-23 appears to produce other cytotoxic compounds.

Typical Fermentation of Albonoursin under Optimum Conditions

When strain KO-23 was cultivated in the medium KP with rotary shaking, considerable amounts of albonoursin were detected not only in the EtOAc extract of culture supernatant but also in the cell extract prepared by extracting cells with MeOH-acetone (1:1) (Fig. 4). The total amount of albonoursin increased abruptly after 2 days of cultivation to reach a maximum (*ca.* 16 mg/liter) that persisted from 3 through 7 days of cultivation. This productivity of albonoursin was 30 times higher than that in the original culture. However, the extracellular/intracellular ratio of albonoursin increased with increasing time of cultivation, suggesting that albonoursin produced inside the cells was gradually transported outside the cells.

Fig. 4. Production of albonoursin under the best culture conditions.

The open square (□), open circle (○) and closed circle (●) indicate the total, intracellular and extracellular amounts of albonoursin.



Under these optimum conditions for albonoursin pro-

duction, strain KO-23 expresses higher enzyme activity for albonoursin biosynthesis. The biosynthetic and bioconversion studies on albonoursin will be separately reported[7].

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