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Selective Production of Fungal Beauveriolide I or III by Fermentation in Amino Acid-supplemented Media

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Beauveriolides I and III, cyclic depsipeptides composed of L-Phe, L-Ala, D-Leu and (3*S*,4*S*)-3-hydroxy-4-methyloctanoic acid, and L-Phe, L-Ala, L-*allo*-Ile and (3*S*,4*S*)-3-hydroxy-4-methyloctanoic acid, respectively, were previously isolated from the culture broth of fungal *Beauveria* sp. FO-6979 as inhibitors of macrophage foam cell formation. To improve the production of these compounds by fermentation, the culture conditions were studied. The production of both beauveriolides was increased five to ten folds by fermentation in the culture media containing tryptone. Further study revealed that addition of L-Leu/L-Ile, but not D-Leu/D-*allo*-Ile, to the culture medium yielded a high and selective production of beauveriolide I or III. As a result, regardless of their separation difficulty due to the similar physico-chemical properties, a large amount of beauveriolide I or III was prepared from the culture broth obtained from L-Leu- or L-Ile-supplemented fermentation, respectively, by one step purification using silica gel column chromatography.

During our screening program for microbial inhibitors of macrophage foam cell formation, beauveriolides I and III were isolated from the culture broth of *Beauveria* sp. FO-6979[1~3]. They are cyclic depsipeptides composed of L-Phe, L-Ala, D-Leu (beauveriolide I)/D-*allo*-Ile (beauveriolide III) and (3*S*,4*S*)-3-hydroxy-4-methyloctanoic acid. Both compounds inhibit lipid droplet formation in mouse macrophages specifically with no cytotoxic effect even at a high dose (20 μM). For further evaluation, a large amount of beauveriolides is necessary. However, there were several problems in the original methods[1] used for beauveriolides production; 1) a long fermentation period (12~16 days) under the static condition, 2) a low titer of beauveriolides production (5~6 μg/ml), and 3) difficulty in the separation of beauveriolides I and III due to their similar physico-chemical properties.

In this paper, methods for the high and selective production of beauveriolide I or III are described. Using these procedures, 800~900 mg of these compounds can be obtained from a 20-liter fermentation broth by one-step silica gel column purification.

Materials and Methods

Fermentation in 12 Different Media

Beauveria sp. FO-6979 was used for the production of beauveriolides. The strain was stored as a spore suspension (10^8 spores/ml) in 50% glycerol at −80°C. The suspension was prepared by washing spores on the surface of a YpSs agar medium (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, and agar 2.0%, pH 6.0) with 5 ml of sterilized water. The spore suspension (40 μl) was transferred into a 50-ml test tube containing 10 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4

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0.1% and agar 0.1%, pH 6.0). The tubes were shaken on a reciprocal shaker for 3 days at 27°C. The seed culture (1 ml) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the production medium. Twelve different production media (A to L) were tested, and their constituents were A (glucose 1.0%, tryptone 0.5%, yeast extract 0.3%, malt extract 0.3% and agar 0.1%, pH 6.0), B (glucose 1.0%, tryptone 2.0%, yeast extract 0.3%, malt extract 0.3% and agar 0.1%, pH 6.0), C (glycerol 1.0%, glucose 2.0%, peptone 2.0%, NaCl 0.1% and trace elements (a solution containing $FeSO_4 \cdot 7H_2O$ 1.0%, $MnCl_2 \cdot 4H_2O$ 1.0%, $ZnSO_4 \cdot 7H_2O$ 1.0%, $CuSO_4 \cdot 5H_2O$ 1.0% and $CoCl_2 \cdot 2H_2O$ 1.0%) 1.0% (v/v), pH 6.0), D (glycerol 3.0%, glucose 1.0%, peptone 0.5%, NaCl 0.2% and agar 0.1%, pH 6.0), E (sucrose 1.0%, glucose 1.0%, corn steep powder 2%, meat extract 0.5%, KH_2PO_4 0.1%, $CaCO_3$ 0.3% and agar 0.1%, pH 6.0), F (sucrose 2.0%, glucose 1.0%, corn steep powder 0.5%, meat extract 0.5%, KH_2PO_4 0.1%, $CaCO_3$ 0.3% and agar 0.1%, pH 6.0), G (glycerol 0.5%, glucose 0.5%, sucrose 0.5%, wheat germ 0.5%, soybean peptide 1.0%, $CaCO_3$ 0.2%, allophane 0.5% and trace elements 1.0% (v/v), pH 6.0), H (glucose 1.0%, L-asparagine 1.0%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05% and yeast extract 0.1% pH 6.0), I (sucrose 2.0%, glucose 1.0%, corn steep powder 0.5%, meat extract 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3%, agar 0.1%, and trace elements 1.0% (v/v), pH 6.0), J (potato dextrose broth 2.4%, malt extract 0.5%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.5% and agar 0.1%, pH 6.0), K (soluble starch 1.0%, lactose 1.0%, soybean meal 1.0%, NaCl 0.3% and $CaCO_3$ 0.3%, pH 6.5), and L (glucose 1.0%, sucrose 1.0% and oatmeal 2.0%, pH 6.0). The flasks were shaken on a reciprocal shaker for 10 days at 27°C.

Fermentation in Amino Acid-supplemented Media

The seed culture (1 ml) was transferred into a 500-ml flask containing 100 ml of medium A. On day 1, an amino acid (0.1% of D-Leu, D-Ile, L-,D-*allo*-Ile, L-,D-Phe or L-,D-Ala, or 0.1, 0.2, 0.3 or 0.5 % of L-Leu or L-Ile) was added to the culture. Fermentation continued for 6 days, and the production of beauveriolides was determined by HPLC as described below.

Quantitative Analysis of Beauveriolides by HPLC

On days 0, 2, 4, 6, 8 and 10, 1 ml of the culture broth was centrifuged to precipitate the mycelial component that was treated with 1 ml of $CHCl_3$-MeOH (2:1) to extract beauveriolides. HPLC was carried out using the HP Series 1100 systems (Hewlett Packard). To determine the amounts of beauveriolides I and III, the samples (1 μl) were analyzed by HPLC under the following conditions; column, Symmetry C_{18} (Waters), 2.1×150 mm; solvent, a 20-minute linear gradient from 5% CH_3CN in 0.05% H_3PO_4 to 100% CH_3CN; flow rate, 0.2 ml/minute; UV detection, 210 nm. Beauveriolides I and III were eluted as peaks with retention times of 16.7 and 17.3 minutes, respectively.

Amino Acid Analysis of Hydrolysates of Beauveriolides by Chiral HPLC

The absolute stereochemistry of amino acid constituents in beauveriolides obtained by amino acid-supplemented fermentation was determined by the previously reported method[2].

Results and Discussion

Fermentation Conditions for Beauveriolides Production

Under the original static-phase fermentation of *Beauveria* sp. FO-6979, beauveriolides I and III were produced in low titers (5~6 μg/ml) after a long incubation time (12~16 days)[1]. To improve their yield and culture conditions, the time course of beauveriolides production in 12 different media under the shaking fermentation was compared (Table 1). The production of both beauveriolides increased dramatically 5 to 10 times higher in media A and B than in the original medium, indicating that tryptone is necessary for high beauveriolides production. Furthermore, their titers reached a maximum on day 6, a much shorter fermentation time than originally (day 14). Among the 12 media, rather high titer of beauveriolide I (41 μg/ml) and the highest titer of beauveriolide III (80 μg/ml) were yielded when fermented in medium A.

Selective Production of Beauveriolide I or III in Amino Acid-supplemented Media

The effect of the addition of Phe, Ala, Ile or Leu to medium A on beauveriolides production was studied, as these compounds consist of L-Phe, L-Ala and D-Leu/D-*allo*-Ile moieties. Either L-Leu, D-Leu, L-Ile, D-Ile L-*allo*-Ile, D-*allo*-Ile, L-Phe, D-Phe, L-Ala or D-Ala (0.1%) was added to medium A on day 1, and the amount of beauveriolides I and III in the culture on day 5 was determined by HPLC. As summarized in Table 2, addition of L-Ala or L-Phe, which are common constituents of beauveriolides, to the medium gave a slight (1.2 folds) or moderate (1.6~2.6 folds) increase in the production of both beauveriolides, whereas addition of D-Ala or D-Phe had no effect. Interestingly, addition of D-Leu or D-*allo*-Ile, another constituent of these

Table 1. Time course of beauveriolides I and III production cultured in 12 different media.

Medium	Beauveriolide I (μg/ml)						Beauveriolide III (μg/ml)					
	Day						Day					
	2	4	6	8	10	14	2	4	6	8	10	14
Original	−	−	0	3	4	6	−	−	0	2	4	5
A	0	0	41	14	2	−	0	0	80	24	5	−
B	0	0	44	14	5	−	0	0	50	23	5	−
C	0	0	49	30	4	−	0	0	34	20	0	−
D	0	0	22	19	39	−	0	0	18	19	36	−
E	0	0	27	18	4	−	0	0	50	28	6	−
F	0	0	4	2	0	−	0	0	7	4	0	−
G	0	0	15	13	6	−	0	0	40	31	13	−
H	0	0	8	4	0	−	0	0	20	9	0	−
I	0	0	0	0	0	−	0	0	0	0	0	−
J	0	0	0	0	0	−	0	0	0	0	0	−
K	0	0	0	0	0	−	0	0	0	0	0	−
L	0	0	0	0	0	−	0	0	0	0	0	−

−, not determined.

compounds, showed almost no influence on the production. However, addition of L-Ile enhanced the yield of beauveriolide III (50→172 μg/ml) and inhibited the production of beauveriolide I (25→5.6 μg/ml), resulting in very selective production of beauveriolide III. Similarly, addition of L-Leu yielded very selective production of beauveriolide I (127 μg/ml *vs* 26 μg/ml for III). The concentration of L-Ile or L-Leu (0.2, 0.3 or 0.5%) was tested. The addition of L-Ile at 0.2% gave the most selective production for beauveriolide III (189 μg/ml *vs* 3.9 μg/ml for I). Similarly, addition of L-Leu at 0.2% yielded the most selective production for beauveriolide I (142 μg/ml *vs* 18 μg/ml for III).

These results strongly suggested that putative beauveriolide synthetase incorporated L-Leu/L-Ile selectively and efficiently, and epimerized it to D-Leu/D-*allo*-Ile to form beauveriolide I /III. In fact, KLEINKAUF and VON DOHREN reported that non-ribosomal peptide synthetase contains amino acid epimerase domains[4~6]. We showed first in this paper that addition of L-amino acid to the culture selectively enhances the production of the depsipeptide containing the D-counterpart as a constituent.

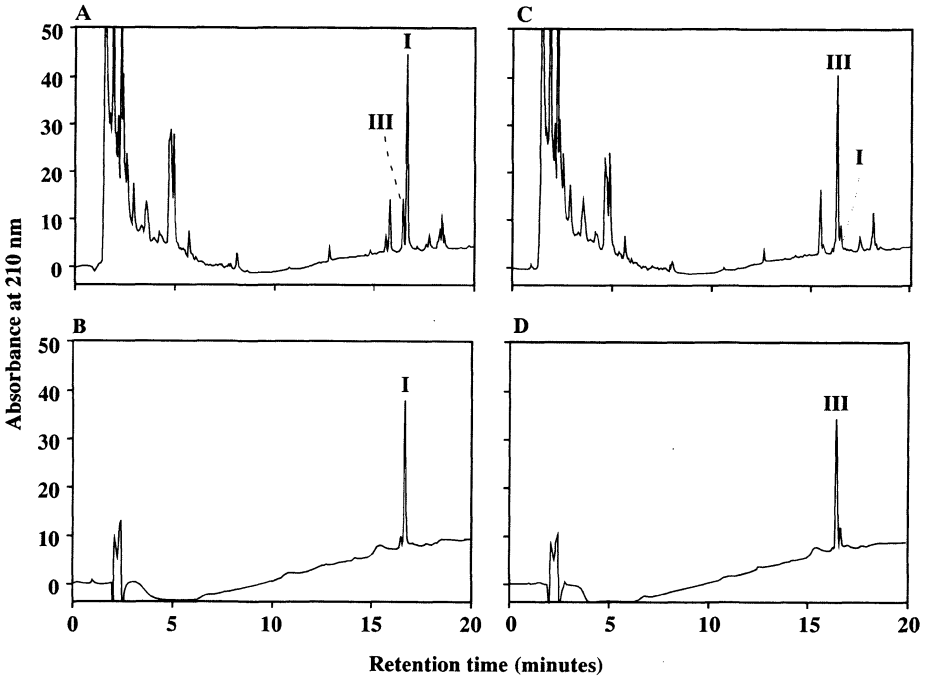
Scaled-up Preparation of Beauveriolide I or III

Based on these findings, a scaled-up fermentation was carried out to obtain a large amount of beauveriolide III. The seed culture (200 ml) was transferred into a 30-liter jar fermenter containing 20 liters of medium A. L-Ile (0.2%) was added to the culture on day 1. Analysis of the culture broth on day 5 by HPLC (Fig. 1C) confirmed that beauveriolide III was produced selectively (178 μg/ml *vs*

Table 2. Production of beauveriolides I and III by amino acid addition.

Medium	Beauveriolide I	Beauveriolide III
	(μg/mL)	
Medium A	25	50
+L-Ile	5.6	172
+D-Ile	25	50
+L-*allo*-Ile	24	42
+D-*allo*-Ile	14	33
+L-Leu	127	26
+D-Leu	24	48
+L-Phe	66	79
+D-Phe	29	46
+L-Ala	30	60
+D-Ala	28	50

Fig. 1. Quantification of beauveriolides I and III by HPLC in the starting crude extracts and the samples after one-step silica gel purification.



Beauveria sp. FO-6979 mycelium fermented in the L-Leu (A)- or L-Ile (C)-supplemented medium was extracted with $CHCl_3$-MeOH solution (2:1). After concentration, the samples (1 μl) dissolved in MeOH were subjected to HPLC. Beauveriolides I (B) and III (D) were obtained by one step purification using a silica gel column chromatography of the $CHCl_3$-MeOH extracts. Each beauveriolide (0.1 μg) dissolved in 1 μl MeOH was subjected to HPLC.

3.6 μg/ml for I). The 5-day old whole broth (20 liters) was centrifuged to collect the mycelium. The mycelial component was treated with 20 liters of $CHCl_3$-MeOH solution (2:1). The extracts were filterated and concentrated to give a brown powder. This powder was then washed with hexane (3 liters) and was centrifuged to give a yellow powder (8.9 g). This powder was dissolved in $CHCl_3$ and was applied on a silica gel (890 g) column that was previously equilibrated with $CHCl_3$. The materials were eluted stepwise with $CHCl_3$-MeOH solutions (5 liters, 100:0, 100:1, 100:2 and 0:100), and each 150 ml of the elution was collected to give a total of 132 fractions. Selected fractions (39th to 74th) were pooled and concentrated *in vacuo* to give beauveriolide III (893 mg) (93% purity, Fig. 1D). From other fractions (53rd to 58th and 75th to 96th), beauveriolide III (2.1 g) of 30% purity was recovered.

Similarly, from the 5-day old broth (Fig. 1A) fermented in L-Leu (0.2%)-supplemented medium A, beauveriolide I (850 mg) (95% purity, Fig. 1B) was purified. Impure beauveriolide I (1.9 g, 28% purity) was also recovered.

The hydrolysates of beauveriolides I and III obtained by fermentation in L-Leu- and L-Ile-supplemented media, respectively, were analyzed to confirm the absolute stereochemistry of the constituent amino acids. The amino acids (L-Ala, L-Phe and L-Leu for beauveriolide I and L-Ala, L-Phe and D-*allo*-Ile for beauveriolide III) were determined, indicating that the same chiral molecules were biosynthesized by this fermentation.

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