A Prolyl Endopeptidase Inhibitor, Propeptin Production in the Various *Microbispora* sp.

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We examined the propeptin production in 10 species of mesophilic *Microbispora*. Three strains which have closely DNA homology to *Microbispora rosea*, *M. rosea* (IFO 14044), *M. rosea* subsp. *nonnitrogenes* (IFO 14045) and *M. indica* (IFO 14879) produced propeptin, other 7 strains did not. The results suggested some relationship between secondary metabolite production and taxonomy.

In the course of screening for a new type of prolyl endopeptidase (PEP) inhibitor from actinomycetes, we have found that *Microbispora* sp. SNA-115 (Fig. 1.) isolated from a soil sample produces a new PEP inhibitor designated as propeptin¹⁾. Propeptin is an atypical cyclic peptide

Fig. 2 Structure of propeptin.

Fig. 1. Cryo scanning electron micrograph of *Microbispora* sp. SNA-115. Medium, Tyrosine agar (ISP No.7); Cultivation, 27°C for 21 days; the bar represents 2 μ m.

antibiotic containing nineteen amino acids which is composed of a ring region with nine amino acids and a side chain region with ten amino acids (Fig. 2.). All amino acids except glycine are L-form. An ANF antagonist, anantin²⁾ and an endotherin antagonist, RES 701-1³⁾, both compounds produced by *Streptomyces* sp. have very similar cyclic structure to propeptin. That is, all compounds have an amide bond between the α -amino group of the amino-terminal Gly¹ and the β -carboxyl group of Asp⁸ or Asp⁹ to make ring structure. We are interested in whether propeptin and/or propeptin type peptide are produced by other species of *Microbispora*. In this paper, we report the production of propeptin in the various mesophilic *Microbispora* sp.

PEP (*Flavobacterium*) and substrate (Z-Gly-Pro-pNA) were purchased from Seikagaku Kogyo Co., Ltd. The strains of *Microbispora* sp. were purchased from IFO (Institute For Fer-

Table 1. Strains used in this study

<i>Microbispora rosea</i>	IFO 14044
<i>Microbispora rosea</i> subsp. <i>nonnitritigenes</i>	IFO 14045
<i>Microbispora indica</i>	IFO 14879
<i>Microbispora parva</i>	IFO 14043
<i>Microbispora chromogenes</i>	JCM 3022
<i>Microbispora diastatica</i>	IFO 14041
<i>Microbispora amethystogenes</i>	IFO 14039
<i>Microbispora amethystogenes</i> subsp. <i>nonreducans</i>	IFO 14040
<i>Microbispora karnatakensis</i>	IFO 14875
<i>Microbispora viridis</i> (<i>Actinomadura rugatobispora</i>)	IFO 14382
<i>Microbispora</i> sp. SNA-115

Fig. 3. A typical time course of fermentation by *Microbispora* sp. SNA-115 and other *Microbispora* sp. (500 ml Erlenmeyer flask). B, broth filtrate; M, mycelium extract; Myc, mycelium.

Fig. 4. HPLC analysis patterns of propeptin, the acetone extracts of the mycelium of *Microbispora indica* and *Microbispora parva* on day 4. Column, Capcell pack (4.6 ϕ x 250 mm); Solvent, 30-60% CH_3CN containing 0.1% TFA (linear gradient, 20 min.); Flow rate, 1ml/min.; Detector, UV(254 nm).

mentation, OSAKA) and JCM (Japan Collection of Microorganisms) as listed in Table 1. *Microbispora* sp. SNA-115 (FERM-P12094) and other strains from slant cultures were inoculated into each 500 ml volume Erlenmeyer flask con-

taining 70 ml of the medium composed of glucose 2 %, soluble starch 1 %, meat extract 0.1 %, dried yeast 0.4 %, soybean flour 2.5 %, NaCl 0.2 % and K_2HPO_4 0.005 %, (pH 6.7), and the flasks were incubated for 10 days at 27°C on a rotary shaker (200 rpm). After two milliliter of the culture was inoculated into a flask containing the same medium and incubated at 27°C continuously for each strain. PEP inhibition activity using the cultured broth was measured as described previously⁴⁾ and isolation procedure of propeptin was the same as that described previously¹⁾. In brief, the acetone extract of mycelium from a flask was extracted with BuOH and evaporated. It was dissolved in MeOH and chromatographed on a silica gel TLC with BuOH : MeOH : H_2O = 4 : 1 : 2. The UV band with PEP inhibition activity (R_f = 0.36) was scraped and crude propeptin was eluted with MeOH. Final purification was carried out by HPLC using ODS column with CH_3CN - 0.1 % TFA (40 : 60). After concentration and lyophilization, propeptin was obtained as a white powder. Propeptin was identified using HPLC, amino acid analysis and PEP inhibition activity.

The typical time course of propeptin production in a 500 ml volume Erlenmeyer flask containing 70 ml of the medium was shown in Fig. 3. The growth curve and the PEP inhibition activity patterns of such strains as *M. rosea* (IFO 14044) and *M. rosea* subsp. *nonnitritogenes* (IFO 14045) were similar to those of *Microbispora* sp. SNA-115. *M. indica* (IFO 14879) was different from the above strains in those patterns, but produced propeptin (Fig. 3.). Though other 7 strains were grown enough in the medium, the PEP inhibition activity was not detected in their culture broth under the cultured conditions employed in this study (Fig. 3.). PEP inhibition activity in the broth filtrate and the acetone extract of the mycelium of the various *Microbispora* sp. on day 4 and DNA homologies of strains of *Microbispora*, which have been reported by Miyadoh *et al.*⁵⁾, were listed in Table 2. Though *M. indica* which has DNA homology of 83 % to *M. rosea* produced propeptin, *M. parva* which has DNA homology of 78 % did not. Other

Table 2. Inhibition activity of prolyl endopeptidase in the strains of *Microbispora* sp.

strain	% Hybridization*	PEP inhibition(%)		propeptin production***
	<i>M. rosea</i>	B**	M**	
<i>Microbispora rosea</i>	100	6	52	+
<i>Microbispora rosea</i> subsp. <i>nonnitritogenes</i>	-	17	70	+
<i>Microbispora indica</i>	83	47	51	+
<i>Microbispora parva</i>	78	14	11	+
<i>Microbispora chromogenes</i>	68	1	0	-
<i>Microbispora diastatica</i>	68	0	3	-
<i>Microbispora amethystogenes</i>	57	0	13	-
<i>Microbispora amethystogenes</i> subsp. <i>nonreducans</i>	-	0	0	-
<i>Microbispora karnatakensis</i>	52	0	27	-
<i>Microbispora viridis</i> (<i>Actinomadura rugatobispora</i>)	7	0	10	-
<i>Microbispora</i> sp. SNA-115	-	20	65	+

* S. Miyadoh et al., J. Gen. Microbiol., 136, 1905(1990).

** B, Broth filtrate; M, Mycelium extract at 4 days.

*** HPLC analysis

strains which had less homology to *M. rosea* than *M. parva* also did not produce propeptin.

HPLC analysis patterns of propeptin, the acetone extract of the mycelium of *M. indica* and *M. parva* on day 4 were shown in Fig. 4. In *M. indica*, two peaks around 15 min were increased on time dependently, but the same peaks were not recognized in *M. parva*. Similar HPLC patterns observed in each the produce and the non-produce strain (data not shown). The broth filtrate and/or the acetone extract of the mycelium of *M. parva*, *M. amethystogenes*, *M. karnatakensis* and *M. viridis* exhibited weak inhibition activity (Fig. 3 and Table 2), but the peak of propeptin was not detected by HPLC analysis. This weak inhibition activity might be derived from non-specific inhibition or disturbance of the absorbance at 410 nm of *p*-nitro-aniline by color of broth filtrate and/or acetone extract of mycelium. These results show that the production of propeptin may be closely related to the taxonomy of *Microbispora* sp.

Ochi *et al.* have reported that the phylogenetic relationships among *Microbispora* sp. as determined by ribosomal protein AT-L30 analysis⁶⁾. *M. rosea* JCM 3006^T and *M. indica* ATCC 35926^T which are the same species as producing

strains have the same electrophoretic mobility (20.0 mm) of AT-L30 protein. *M. diastatica* JCM 3023^T, *M. amethystogenes* JCM 3021^T, *M. parva* JCM 3024^T and *M. karnatakensis* ATCC 35927^T which are the same species as non-producing strains have the different electrophoretic mobility of them (5.0-9.3 mm). Wong *et al.* also examined 16S rRNA gene sequence-based phylogenetic analysis of *Microbispora* sp.⁷⁾. Though they reported the analysis of *M. parva*, *M. diastatica*, *M. chromogenes* and *M. amethystogenes* which are the same species as non-producing strain, but no report about *M. rosea* and *M. indica* which are the same species as producing strain. Comparison of 16S rRNA gene sequence between the producing strains and non-producing strains might support our results.

We are now interested in the biosynthesis gene of propeptin in *Microbispora* sp. and participation of propeptin in the life cycle of *Microbispora*. We will further examine whether the non-producing strains such as *M. parva* have the gene of propeptin biosynthesis, and other genus excluded *Microbispora* and *Streptomyces* produce the propeptin-type peptide (amide bond between the α -amino group of the amino-terminal Gly¹ and the β -carboxyl group of Asp⁸ or Asp⁹, all

amino acids are L-type) in the future.

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