Note

Biological Activity of Antifungal Substances Produced by *Bacillus subtilis*

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Two fungicidal substances, Y-9 and Y-10, were isolated from the culture filtrate of *Bacillus subtilis* TM-105 during our screening for antimicrobial substances against phytopathogenic fungi. They were identified as lipopeptide antibiotics plipastatin A1 and B1,¹⁻⁴⁾ respectively. Plipastatins have been reported to be new inhibitors of phospholipase A₂, but their fungicidal activity has yet to be clarified. We report here the result of our investigation on the fungicidal activity of plipastatins.

1. Purification of Antifungal Substances

Bacillus subtilis TM-105 was fermented at 30°C for 120 hr in an Erlenmeyer's flask containing one liter of medium composed of glucose (1%, w/v), peptone (0.5%), KH₂PO₄ (0.01%) and MgSO₄·7H₂O (0.005%), pH 6.8. The fermentation broth was centrifuged at 12,000×g for 30 min. The supernatant was adjusted to pH 3.0 with 6 N HCl and stood at 4°C for an hr. The precipitate was collected by centrifuge (2000×g, 15 min), and extracted with methanol. The methanol extract was filtered and concentrated *in vacuo* to dryness. The residue was applied to silica gel TLC with a solvent system of 1-butanol/acetic acid/water (65:10:25, v/v/v). Active spots were scraped off and extracted with methanol. Further purification was done by HPLC using an ODS column (Senshu Pac ODS-H-4251) with a solvent of methanol/water/10% diethylamine-formic acid buffer (8:1:1, v/v/v, pH 7.5), resulting in separation of two biologically active peaks. Final purification was done on a Sephadex LH-20

column using methanol as a solvent.

Chemical and physical properties of the two active substances, Y-9 and Y-10, were extensively studied and proved to coincide with plipastatin A1 and B1, respectively.

2. Fungicidal Activities

Plipastatin A1 and B1 inhibited the growth of *Pyricularia oryzae* and some plant pathogenic fungi, as shown in Table 1. The inhibitory activity on *Botrytis cinerea* (gray mold pathogen) and *Pyricularia oryzae* (rice blast pathogen) was accompanied by the swelling of the mycelia. The blast fungus was particularly sensitive to the antibiotics, with mycelial swelling at 0.78 µg/ml of plipastatin A1.

Protective activity was tested against rice blast and Helminthosporium leaf spot. Rice seedlings (cultivar: Aichiasahi) of four leaf stage grown in plastic pots were used to examine the disease control activity of samples. Three pots,

Table 1 Antimicrobial activity of plipastatin A1 and B1 against various types of microbes.

Organisms	Diameters of inhibition zones (mm)	
	Plipastatin A1 ^{a)}	Plipastatin B1
<i>Escherichia coli</i> AB 1157	0	0
<i>Salmonella typhimurium</i> TV 119	0	0
<i>Staphylococcus aureus</i> IFO 12732	0	0
<i>Pseudomonas aeruginosa</i> IFO 13130	0	0
<i>Xanthomonas oryzae</i> IFO 3312	0	0
<i>Erwinia carotovora</i> IFO 12380	0	0
<i>Alternaria mali</i> IFO 8984	18	19
<i>Botrytis cinerea</i> IFO 5365	22	20
<i>Pyricularia oryzae</i> IFO 5994	24	21
<i>Candida albicans</i> IFO 1594	0	0
<i>Chlorella vulgaris</i>	0	0

^{a)} Concentrations of plipastatin A1 and B1 solutions, in which paper disks were dipped, were 250 ppm and 115 ppm, respectively.

Table 2 Protective values against rice blast and Helminthosporium leaf spot.

	Concentra- tions (ppm)	Protective values (%)	
		Rice blast	Helmintho- sporium leaf spot
Broth	—	83	—
Methanol extract	100	83	69
	50	29	46
Plipastatin A1	100	42	25
Plipastatin B1	100	—	57
Iturin A	100	25	0

each with ten seedlings, were assigned to each test plot. Samples were diluted to given concentrations and sprayed with a spreader (Shin-guramin®) onto rice seedlings. Conidial suspensions of *Pyricularia oryzae* P-2 or *Cochliobolus miyabeanus* were sprayed onto rice seedlings 4 to 5 hr after sample application. The number of lesions appearing on the leaves was counted after incubation for 24 hr at high humidity (100% relative humidity, 25°C) followed by incubation for 5 days in a growth chamber. The protective values were obtained by the following equation.

Protective value (%)

$$= 100 \times \{1 - (\text{mean No. of lesions/leaf on treated seedlings}) / (\text{mean No. of lesions/leaf on untreated seedlings})\}$$

The methanol extract from the acid precipitate of culture filtrate containing plipastatin A1, B1 and iturin A⁵⁾ exhibited high protective values against rice blast and Helminthosporium leaf spot in the pot test (Table 2). However, the activities of plipastatin A1, B1 and iturin A against rice blast and Helminthosporium leaf spot were lower at a concentration of 100 ppm. This shows that the high protective values of the methanol

extract were a result of joint action of plipastatin A1, B1 and iturin A.

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要 約

枯草菌の産生する抗糸状菌物質の生物活性

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Bacillus subtilis TM-105 株の培養液より抗糸状菌物質 Y-9, Y-10 を単離した。両物質の構造解析の結果, リポペプチド系抗生物質 plipastatin A1 および B1 と同定した。生物活性を検討したところ, *in vitro* でリンゴ斑点落葉病菌, 野菜灰色かび病菌, イネいもち病菌に抗菌活性が認められた。また, ポット試験では, イネいもち病およびごま葉枯病に病害制御活性が認められたが, 精製各段階の検体を供試したところ, 同菌によって同時に生産される iturin A と共存することにより, 高い防除効果を示すことを見いだした。