Rapid Paper

Hyphal Extension Inhibitors I and II with Similar Inhibitory Spectra to Validamycin, Isolated from Hyphae of *Rhizoctonia solani*

Motoo SHIBATA, Yutaka KIDO,
Yoshiteru HONDA and Kenshi SHIMIZU

Faculty of Pharmaceutical Sciences,
Kumamoto University,
Kumamoto 862, Japan

Received November 14, 1988

A hyphal extract was obtained by methanol extraction of a hyphal cake of *Rhizoctonia solani* culture. The crude hyphal extension inhibitor (HE-inhibitor), which only inhibits hyphal extension, i.e., without growth inhibition, was extracted with ethyl acetate, at pH 9.5, from the aqueous hyphal extract. The HE-inhibitor was further purified by silica gel column chromatography with a solvent mixture of benzene and acetone, and then separated into HE-inhibitors I and II. HE-inhibitor I, which highly inhibited the hyphal extension, with characteristic curling of the hyphal tips, was isolated as a colorless syrup, whereas HE-inhibitor II, with weak activity, was isolated as colorless needles: mp 187°C; C, 80.38, 79.14; H, 4.36, 4.96; N, 16.10, 15.94%. HE-inhibitors I and II exhibit no antibiotic activity against various fungi, yeasts and bacteria, but inhibit the hyphal extension of *Rhizoctonia solani* R-44 at 0.1 µg/ml and 5.0 µg/ml, respectively. Their inhibitory spectra resemble that of validamycin, though both inhibitors are less active than validamycin.

The hyphal extension factor (HE-factor), which antagonizes validamycin and is thought to be involved in hyphal extension, and the hyphal extension inhibitor (HE-inhibitor), which antagonizes the action of the HE-factor, are both present in the hyphae of *Rhizoctonia solani*.^{1,2)} The HE-inhibitor was found to consist of two components (HE-inhibitors I and II), on fractionation by silica gel column chromatography. Both HE-inhibitors I and II only inhibit the extension of hyphae of *R. solani*, i.e., without growth inhibition, the former causing characteristic curling of the hyphal tips and the latter inhibiting mainly the extension of the main hyphae.³⁾ We describe

here the isolation and some properties of HE-inhibitors I and II.

Materials and Methods

Microorganism. *Rhizoctonia solani* Kühn R-44, which was kindly donated by the Kyoto Herbal Garden, Takeda Chemical Co., was used throughout this work. The strain was kept at 4°C, after 5-days incubation at 27°C, on potato sucrose agar.

Preparation of a hyphal extract of *R. solani*. *R. solani* was grown in a 30-liter jar-fermentor or a 1000-liter tank-fermentor containing 15 l or 500 l of medium S (glucose 2%, starch 3%, corn steep liquor 1%, soybean flour 1%, peptone 0.5%, NaCl 0.3% and CaCO₃ 0.5%, pH 7.0), respectively. After 2-days culture at 27°C, a hyphal cake was obtained by filtration. The hyphal cake was extracted with 10 volumes (v/w) of 70% methanol under reflux for 1 hr and then after filtration the resultant extract was concentrated *in vacuo* to remove methanol.

Determination of the minimum effective concentration (MEC) for the inhibition of hyphal extension. The dendroid test method for the determination of validamycin was applied, using (serial dilutions of) the HE-inhibitor instead of validamycin.¹⁾ The minimum effective concentration was defined as the concentration of the highest dilution at which hyphal extension was inhibited, compared with the hyphal extension in a control culture (plate culture).

Modified dendroid test method. In order to detect the activity of a small amount of the HE-inhibitor the dendroid test method was modified as follows. The paper disk, after dipping in a sample solution, was placed on a glass disk, previously laid on a water agar plate. On this paper disk, an agar disk (inoculum) of *R. solani* was placed, followed by incubation at 27°C for 24~48 hr. Then, the length of hyphal extension from the inoculum was compared with that in a control culture (without HE-inhibitor).

Results and Discussion

The crude HE-inhibitor was isolated as shown in Fig. 1 from a hyphal extract, previously prepared as described under **Materials and Methods**. The crude HE-inhibitor was further purified by silica gel column chromatography with solvent mixtures (benzene-acetone=9:1, 7:3, 5:5) and then separated into HE-inhibitors I and II. HE-inhibitor I, which highly inhibits the hyphal extension, was isolated as a colorless syrup from the eluate with benzene

Fig. 1. Isolation of HE-inhibitors I and II.

Fig. 2. UV Absorption Spectrum of HE-inhibitor II (MeOH).

and acetone (9:1), whereas HE-inhibitor II, with weak activity, was isolated as colorless needles from the eluate with benzene and acetone (7:3). Further collection and purification of HE-inhibitor I are now in progress. 14.3 mg of HE-inhibitor II was obtained from 500 l of culture broth (yield, 0.06%). Some properties of HE-inhibitors I and II are summarized in Table I. Based on the results of elemental analysis and the FAB-MS spectrum ($M+H$ m/z : 183), the molecular formula for HE-inhibitor II was presumed to be $C_{12}H_{10}N_2$. The UV and IR spectra of HE-inhibitor II are shown in Figs. 2 and 3. HE-inhibitors I and II showed no antibiotic activity against various fungi, yeasts and bacteria. HE-inhibitors I and II, however, inhibit the hyphal extension of *R. solani*, without growth inhibition, followed by curling or abnormal branching at the tips. As shown in

Table I. SOME PROPERTIES OF HE-INHIBITORS I AND II

	I	II
Appearance	Colorless syrup	Colorless needles
Melting point (°C)	—	187
Analysis (%)	C, 47.79 H, 8.01 N, 3.24	C, 80.38, 79.04 H, 4.36, 4.96 N, 16.10, 15.94
$[\alpha]_D^{20}$	—	0 ($c=1.0$, EtOH)
UV $\lambda_{\max}$ (MeOH) nm ($E_{1\%}^{1\text{cm}}$)	—	214 (940), 235 (1550) 283 (470), 290 (730)
Color reaction (+)	Ninhydrin, anthrone, Molisch	Dragendorff, tertiary amine, KMnO_4
(-)	Fehling, Elson-Morgan	Ninhydrin, Na-nitroprusside, FeCl_3
Solubility	Soluble in H_2O , MeOH, EtOH, Me_2CO , EtOAc, C_6H_6 Insoluble in H_2O	Soluble in MeOH, EtOH, Me_2CO , EtOAc, C_6H_6 Insoluble in H_2O

Table II. REPRESSION OF HYPHAL EXTENSION OF VARIOUS FUNGI BY VALIDAMYCIN, AND HE-INHIBITORS I AND II

Test organism	Minimum effective concentration ($\mu\text{g}/\text{ml}$)		
	Validamycin	HE-inhibitor I	HE-inhibitor II
<i>Rhizoctonia solani</i> R-44	0.001	0.1	5
<i>Rhizoctonia solani</i> R-51	0.005	0.2	10
<i>Rhizoctonia fragariae</i>	0.02	0.2	50
<i>Sclerotium hydrophilum</i> IFO-5293	0.05	0.5	50
<i>Aspergillus niger</i> IFO-4066	> 500	> 500	> 500
<i>Aspergillus oryzae</i> IFO-4075	> 500	> 500	> 500
<i>Penicillium chrysogenum</i> IFO-4626	> 500	> 500	> 500

Fig. 3. IR Absorption Spectrum of HE-inhibitor II (KBr).

A

B

C

Fig. 4. Hyphal Tips of *Rhizoctonia solani* ($\times 150$).

A: Normal Hyphae.

B: Hyphae inhibited by HE-inhibitor I (20 $\mu\text{g/ml}$).

C: Hyphae inhibited by HE-inhibitor II (20 $\mu\text{g/ml}$).

Table II, they also inhibit the hyphal extension of some kinds of *Basidiomycetes* or *Ascomycetes*, besides *R. solani*. The similarity of their

inhibitory spectra to that of validamycin is quite interesting, though the orders of their activities are considerably different. The hyphal tips of *R. solani*, treated with 20 $\mu\text{g/ml}$ of HE-inhibitors I and II, are shown, respectively, in Fig. 4, compared with those of normal hyphae. Sorbose and validamycin A are known to reduce the extension of hyphae of *R. solani*.⁴⁾ Besides these exogenous substances, there have been a few reports of an auto-inhibitor controlling hyphal growth. Reissig⁵⁾ proposed that a galactosaminoglycan produced by *Neurospora crassa* stops hyphal extension. Similarly, a thermo-labile inhibitor produced by a particular mutant (*smco-9*) of *N. crassa* is known to change its morphology (reduced hyphal extension).⁶⁾

Glucosamine,^{7,8)} which is an intermediate of UDP-glucosamine, was recently found to inhibit not only the extension of *Coprinus lagopus* hyphae but also that of *Pyricularia oryzae* P₂ hyphae. These substances are quite interesting in respect to endogenous inhibitors, different from exogenous inhibitors, sorbose and validamycin. The HE-inhibitor, as already mentioned above, is distinct from these endogenous and exogenous inhibitors. The collection of HE-inhibitor II and elucidation of its chemical structure are now in progress, and the results will be published in the near future.

Acknowledgments. We wish to thank Takeda Chemical Industries Co., Ltd. for culturing *R. solani* in fermentors, and also wish to thank Miss M. Hamashima, Mr. S. Uchida and Mr. H. Ohsako for their technical assistance.

References

- 1) M. Shibata, M. Uyeda and K. Mori, *J. Antibiot.*, **33**, 679 (1980).
- 2) M. Shibata, M. Uyeda and K. Mori, *Agric. Biol. Chem.*, **44**, 2241 (1980).
- 3) M. Shibata and K. Mori, Symposium papers, 5th Symposium on Microbial Sciences, Pharmaceutical Society of Japan, Hiroshima, 1980, p. 156.
- 4) A. P. J. Trinci, *Experimental Mycology*, **9**, 20 (1985).
- 5) J. L. Reissig, *Current Topics in Microbiology and Immunology*, **67**, 43 (1974).
- 6) T. Abramsky and E. L. Tatum, *Biochim. Biophys. Acta*, **421**, 106 (1976).

- 7) D. Moore and G. R. Stewart, *J. Gen. Microbiol.*, **71**, 333 (1972). 8) S. Kamiyama and H. Tanaka, *Agric. Biol. Chem.*, **46**, 1225 (1982).
-