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Short Communication: Bioactive Materials

Extraction and Purification of an Antifungal Antibiotic Saccharide from *Bacillus* sp.

Jae Hong Yoo*

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Abstract An antifungal antibiotic was extracted three times using *n*-butanol from the culture broth of *Bacillus* sp. Bioassay-guided column chromatography with silica gel and Sephadex LH-20 yielded 62 mg of the original active compound from 1 L of culture broth. The minimal inhibitory concentration values were 25 and 50 μg/mL against *Pyricularia oryzae* and *Pellicularia filamentosa*, respectively. Based on results obtained from the analysis of the structure of the antibiotic using MS, NMR, and IR spectroscopy, the antifungal antibiotic was shown to consist of only six of fructose.

Keywords antifungal · *Pellicularia filamentosa* · *Pyricularia oryzae* · saccharide

Numerous studies have been performed to evaluate the efficiency of microorganisms as feed additives for various applications of economic interest in the poultry industry. Antibiotic microbes such as *Bacillus* sp. are widely used in the poultry industry. The idea of using microbes in organic agriculture is based on the enhancement of economically important traits after the addition of microbes to the main materials. The main function of microbes as well as other antibiotics in rice is the secretion of metabolites, particularly antifungal compounds.

A volume of 250 mL *of Bacillus sp.* in optimal medium (3.0% soluble starch, 0.8% yeast extract, 0.2% KCl, pH 8.0) was cultivated in a 1 L flask with 1% at 37°C for 72 h. The cultures were centrifuged at 12,000×*g* for 20 min, and the resulting supernatant was extracted with *n*-butanol three times. The butanol layer was decolorized with charcoal and concentrated *in vacuo*. The residue was redissolved in water and lyophilized to get crude powder. The crude powder was subjected to chromatography on silica gel. The major active compound was found in the MeOH : EtOH (1:1, v/v) eluate. This fraction was subjected to chromatography on the Sephadex LH-20 resin and eluted with MeOH:dichloromethane (5:1, v/v). After evaporation of the solvent *in vacuo*, a yellow powder was obtained.

The antifungal activity of the compound was assessed using *Pyricularia oryzae* IFO 30517 and *Pellicularia filamentosa* IFO 8985 by using the cup method (Iwasa et al., 1971). The melted bottom-layer medium (0.1% sucrose, 1.0% beef extract, 1.0% peptone, and 0.8% agar) was maintained either at 45–55°C with 0.5 mL of the *P. oryzae* IFO 30517 hyphal suspension added, or at 30°C with 0.5 mL of the *P. filamentosa* IFO 8985 hyphal suspension added, and then incubated for 48 h. The plate was overlaid with 10 mL of melted upper-layer medium (0.25% sucrose, 0.1% NaCl, 0.45% peptone, 1.2% agar). After the medium solidified, the cup (6 mm I.D.×10 mm height) was placed on the surface of the upper layer and a solution of test materials was added inside the cup. The inhibition zone was measured after incubation for 48 h at 30°C. The minimal inhibitory concentration was determined using the serial agar dilution method.

For structure identification, the isolated compound was hydrolyzed with 1 N HCl for 10 h at 100°C and was neutralized with 1 N NaOH. The reaction mixture was developed by thin layer chromatography (TLC) with a developing solution consisting of *n*-BuOH : AcOH : H_2O (4:1:5, v/v). The spot was observed after spraying with alkaline $KMnO_4$ solution. Seliwanoff reagent was added to the hydrolysate, fructose, and glucose solutions, and the color change was evaluated.

The hydrolysate and standard sugar were injected into a XBridge Amide high performance liquid chromatography (HPLC) column (Waters, USA), with a refractive index detector, following filtration with a membrane filter. The column used was specific for carbohydrate analysis. The mobile phase was 85:15

J. H. Yoo
National Institute of Agricultural Sciences and Technology, Rural Development Administration, Suwon 441-707, Republic of Korea

*Corresponding author (J. H. Yoo: yj7915@korea.kr)

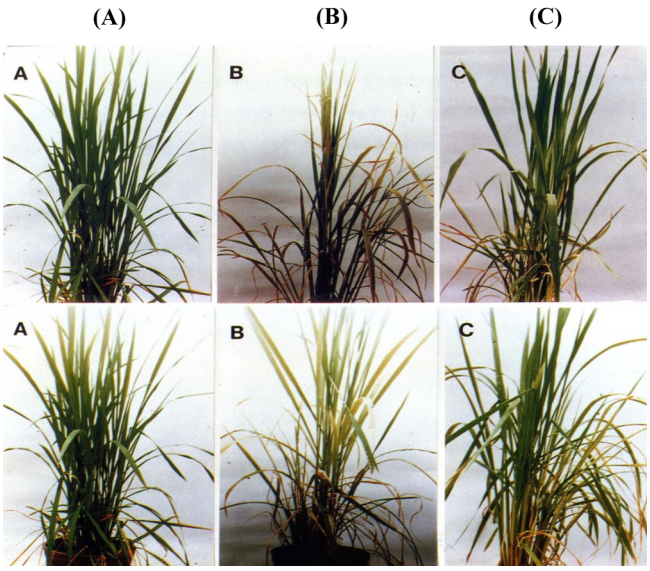

Fig. 1 *In vivo* antibiotic effects of the isolated compound on plant pathogenic microorganisms Top: Effect on *Pyricularia oryzae*; A: plants that were not inoculated with any pathogens, B: plants infected with *Pyricularia oryzae*, C: Plants inoculated with *Pyricularia oryzae* and treated with antibiotic (50 μg/mL). Bottom: Effect on *Pellicularia filamentosa*; A: plants that were not inoculated with any pathogens, B: plants infected with *Pellicularia filamentosa*, C: Plants inoculated with *Pellicularia filamentosa* and treated with antibiotic (100 μg/mL).

(acetonitrile : water, v:v) at a flow rate of 2 mL/min. The *n*-butanol extract from culture broth of *Bacillus* sp. showed an inhibition zone of 34 mm for *P. oryzae* and 31 mm for *P. filamentosa*. After purification with silica gel and Sephadex LH-20 column chromatography, one spot was shown by TLC. The purification yield was 29% and the purification fold was 12.

Inhibition zones of the isolated compound for *P. oryzae* were 15.2, 19.7, 24.5, 28.1, and 32.2 mm at concentration of 62.5, 125, 250, 500, and 1000 μg/mL, respectively. Inhibition zones for *P. filamentosa* were 11.4, 15.5, 20.5, 24.2, and 28.2 mm at concentration of 62.5, 125, 250, 500, and 1,000 μg/mL, respectively.

The MIC of the isolated compound was 25 μg/mL for *P. oryzae* and 50 μg/mL for *P. filamentosa*. Polyoxin showed a MIC of 6.25 μg/mL and 1.6 μg/mL for *P. oryzae* and *P. filamentosa*, respectively (Isono et al., 1986). Nishizawa et al. (1984) reported that dapiramycin had a MIC of 400 and 100 μg/mL for *P. oryzae* and *P. filamentosa*, respectively. There are many antifungal compounds for these two particular fungi that have been reported, such as octacosamicin (Dobashi et al., 1988), fengycin-A (Vanittanakom et al., 1986) and KRF-001 (Kim et al., 1991). The MIC of the isolated compound was comparable to those of these compounds.

From the TLC results following hydrolysis of the isolated compound, there was one spot at Rf =0.30, the same as fructose. The Seliwanoff test is a chemical test that is used to distinguish between aldose and ketose sugars. The isolated compound showed a positive result from the Seliwanoff test, indicating that this chemical contains fructose, not glucose. The hydrolysate from the isolated antifungal compound, fructose, and hydrolyzed trihexocin were injected into the HPLC column. Trihexocin is a tri-saccharide consisting only of fructose. Each sample showed a peak at the same retention time.

The UV spectrum of the antibiotic showed no absorption from 200 to 400 nm. The isolated compound had strong, broad absorption at frequencies of 2800–3700 and 1100–700 cm^{-1}, and the IR spectrum indicated that there were no specific functional groups, such as an aromatic ring, except a hydroxyl (OH) group (Pretsch et al., 2000) present. MS analysis of the isolated antibiotic showed a M^+ peak at m/z 989. This matched with the mass of a hexamer of sugar. ^{13}C NMR spectrum showed six anomeric carbons at δ of 100.63, 100.50, 100.43, 100.31, 96.63, and 92.74 ppm. Based on these results, the antifungal compound produced from the *Bacillus sp.* is a homo-oligosaccharide consisting of only six fructoses.

This chemical has *in vivo* antifungal activity against *P. oryzae* and *P. filamentosa* in rice plants at concentrations of 50 and 100 μg/mL, respectively. This compound could be useful for agricultural purposes.

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