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Purification and Identification of an Antibacterial Metabolite Produced by the Biocontrol Agent, *Streptomyces sioyaensis* Suppressing *Erwinia carotovora* the Causative of Potato Soft Rot

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ABSTRACT

Antibacterial substance produced by *Streptomyces sioyaensis*, obtained from soil and active as a biological control agent against *Erwinia carotovora* sp. *carotovora*, was extracted, isolated and purified to determine the mechanism of disease suppression by this strain. Cultures of *S. sioyaensis* were extracted with chloroform. Both the culture filtrate and the chloroform extract of the strain showed strong growth inhibition against phytopathogen *E. carotovora* sp. *carotovora* The chloroform extract of starch-nitrate broth media of *S. sioyaensis* yielded a white powder antibacterial metabolite by chromatographic technique and was identified as 2-(p-methoxybenzyl)-3,4-pyrrolidinediol-3-acetate on the basis of spectral data. The structure of the bioactive substance was determined by elemental analysis and by ultraviolet (UV), Infra-red (IR), nuclear magnetic resonance (NMR) and mass spectra. From elementary analysis and mass spectroscopic measurements, the molecular formula appeared to be $C_{14}H_{19}O_4N$. The physico-chemical characterization including molecular formula revealed that the isolated antibacterial agent was similar to anisomycin. The bioactive substance showed high antibacterial activity against *E. carotovora* sp. *carotovora*, the causative agent of potato soft rot. This substance may be involved in disease suppression by *S. sioyaensis*.

Key words: Anisomycin, antibacterial, biological control, *Erwinia carotovora*, potato, soft rot, *Streptomyces sioyaensis*.

INTRODUCTION

Soft rot of potato is one of the most important diseases caused by *Erwinia carotovora* (Perombelon and Kelman, 1980). Several organisms antagonistic to *E. carotovora* have been studied for biocontrol of this pathogen in the laboratory, including *Bacillus subtilis* and *Pseudomonas fluorescens* (Kloepper and Schroth, 1981; Kloepper, 1983; Xu and Gross, 1986 and Gnanamanickam, 2002). *Streptomyces sioyaensis* suppressed soft rot of potato and was more effective than other strains in greenhouse tests. In addition it has the ability to colonize the rhizosphere of potato and to inhibit bacterial growth *in vitro* (Mansour *et al*., 2008).

The Actinomycetes and in particular the genus *Streptomyces* have been identified as one of the most potent sources for the production of various antibiotics which are used therapeutically (Waksman, 1962). *Streptomyces* species are heterotrophic feeders, and they can utilize both simple and complex molecules as nutrients. About three-fourths of the *Streptomyces* species may produce antibiotics (Waksman, 1962).

Antibiotic production by some bacteria plays a major role in disease suppression, including *Pseudomonas* and *Bacillus* (Fravel, 1988; Homma *et al*., 1989; Dowling and O'Gara, 1994 and Yu and Sinclair, 1997). Only a few antibiotics produced by some strains were isolated and identified, and their role in biological control has been studied (Refaat, 2007; Kavitha *et al*., 2010 and Zhao *et al*., 2010). Several strains were reported as effective agents for the control of plant pathogens. Antibiotic production may play an important role in their biocontrol activity (Vanneste *et al*., 1992 and Yu *et al*., 2002). Mansour *et al*., (2008) showed that *S. sioyaensis* inhibited *E. carotovora* suggesting that antibiotic production was involved in disease suppression. Identification of the antibiotics produced may improve our standing of the mechanism involved in this and other biocontrol systems.

The goal of this study was to purify and identify certain antibiotics produced by *S. sioyaensis* which are responsible for the *in vitro* inhibition of *E. carotovora*.

MATERIALS AND METHODS

Bacterial strain and production of antibacterial compounds

S. sioyaensis was isolated from a soil sample collected from the rhizosphere of healthy potato plants at Mansoura, Egypt by serial dilution method using starch-nitrate agar medium. *S. sioyaensis* was identified depending on the morphological, cultural, and physiological properties, using all media and methods of International *Streptomyces* Project (ISP) as described by Shirling and Gottlieb (1966). Details of isolation and identification were presented in the paper (Mansour *et al*., 2008). For production of the antibacterial agent, a loopful of *S. sioyaensis* cells from a slant culture of fresh starch-nitrate agar was inoculated into a 500-ml flask containing 100 ml starch-nitrate broth. The flask was incubated on a rotary shaker at 200 rpm for 7days at 28°C.

Extraction of the antibiotic from the culture supernatant using different solvents

As maximum antibiotic production was observed on the 7th day of incubation (Mansour *et al.*, 2008), fermentation was terminated on the 8th day and the broth was centrifuged at 10.000 rpm for 20 min to separate the mycelial biomass. Different solvents were used and tested for the extraction of the antibiotic from the culture supernatant. The solvents used were chloroform, ethyl acetate, diethyl ether and n-butanol to determine the best solvent for extraction of the antibiotic from the culture supernatant. The solvent was added to the supernatant in 1:1 proportion. Solvent-supernatant mixture was shaken vigorously for 15 min. for complete extraction, and then allowed to stand for 10-15 min. The solvent was separated from the broth by separating funnel. All the obtained extracts were concentrated by evaporation to the least volume and then assayed for their antibacterial activities, using the respective solvent as control, by agar well diffusion method (Augustine *et al.*, 2004).

Separation of the antibiotic from the solvent

The solvent, chloroform was evaporated from the crud extract by subjecting the sample to rotating evaporator at 40°C at 50 rpm under vacuum. The white substance obtained was dissolved in chloroform and concentrated, until the crude antibiotic powder was obtained. The crude antibiotic was collected and the obtained residue was subjected to purification.

Purification of antibiotic

The crude antibiotic was tested for number of components present by using precoated thin-layer chromatography plates (G 60) using chloroform: methanol (19:1 vol/ vol) solvent system. Spots were detected by UV light at 254nm. The different spots obtained were scratched, eluted in chloroform and tested individually for its antibacterial activity against *E. carotovora*, using the filter paper disc method. The bioactive spot was loaded again on TLC plates using the same solvent system to confirm its purity. The bioactive fraction was selected for further study, including chemical and spectrometrical analysis.

Characterization of the antibacterial agent

The pure antibacterial agent was identified by ultra-violet (UV), Infra-Red (IR), proton nuclear magnetic (H-NMR) spectra and elementary analysis as reported by El-Sherbiny and Refaat, (2006) and Refaat (2007).

RESULTS AND DISCUSSION

Actinomycetes have been recognized as potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to human. They have provided about two-thirds of the naturally occurring antibiotics discovered. It has been estimated that the genus *Streptomyces* might produce at least 100.000 new compounds of biological interest (Watve *et al.*, 2001; El-Sherbiny and Refaat, 2006; Refaat, 2007 and Kavitha *et al.*, 2010). Several authors have suggested that production of antibiotics could be involved in biological control of plant diseases (Vanneste, *et al.*, 1992; Yu, *et al.*, 2002; Haas and Keel, 2003; Augustine, *et al.*, 2005 and Zhao *et al.*, 2010). Mansour *et al.*, (2008) mentioned that out of 40 actinomycetes, 5 % (8 isolates) exhibited activity against *E. carotovora*. One promising isolate (isolate no.15), was identified as *S. sioyaensis* and selected for further studies for its strong antibacterial activity.

Extraction of the antibacterial metabolite from the culture filtrates of *S. sioyaensis*

Different solvents were used and tested for the extraction of the antibacterial metabolite produced by *S. sioyaensis*. The results in table (1) show that maximum antibacterial activity was observed in residue, extracted by chloroform.

Table (1): Antibacterial activities of the antibacterial metabolite extracted from *Streptomyces sioyaensis* by different solvents

Solvent	Diameter of inhibition zone (mm) by *	
	Organic layer	Aqueous layer
Chloroform	30.0	0.0
Diethyl ether	20.0	35.0
Ethyl acetate	7.0	22.0
n-butanol	24.0	26.0

*The recorded value is the mean of 3 replicates

Detection, separation and purification of the antibacterial agent by TLC

The fractions collected by silica gel chromatography (TLC) technique were checked for their antibacterial activity. One antibacterial compound was isolated and purified. The antibacterial activity was determined according to the agar diffusion assay. The active fraction has R_f value of 0.31 using chloroform: methanol solvent system (19: 9 vol/vol). The active fraction was further characterized by physical, chemical and spectrometric methods.

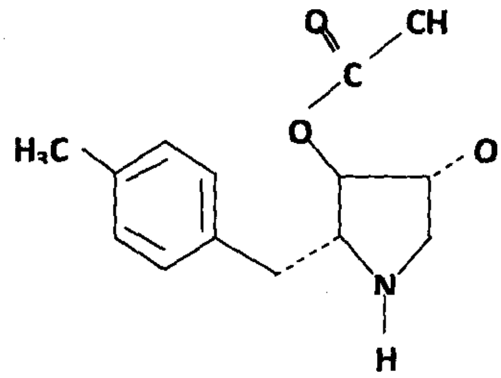

Fig. (1): Structure of the anisomycin antibiotic (2-(p-methoxybenzyl)-3,4- pyrrolidinediol-3-acetate).

Characterization and identification of the antibacterial agent

Elemental analysis of the compound

The elemental analysis showed the presence of carbon 63.53 %, hydrogen 7.24 %, nitrogen 5.21 % and oxygen 24.02 %. No sulphur or halogens were detected. The antibacterial agent is a white colored powder.

Structural analysis of the antibacterial agent

The UV spectral data exhibited strong absorption at max 231, 262, 289, 314 and 384 indicating an aromatic molecule. The IR spectrum exhibited absorption bands at 3420 which indicates hydroxyl group and 2852 indicating stretchy C-H, aromatic, 1733 indicating CO carbonyl, 1457, 1376, 1277 and 1164 indicating C-O-C. Nuclear magnetic resonance (1HNMR, 300 MHz) spectrum of the antibiotic in CDCL3 shows AABB system at 7.029, 7.001 and 6.739, 6.711, with coupling constant J = 8.5 Hz indicating the presence of 1.4 dissubstituted benzene ring. The spectrum showed a singlet at 3.73 indicating the presence of a methoxy group and signals at 2.0, 2.0 and 2.01 for protons of OH, NH and H3CCO, respectively. The other signals at 3.91, 3.72, 3.03 and 2.7 represented the residual aliphatic protons. Obtained data indicated that the compound probably had a molecular weight formula of $C_{14}H_{19}O_4N$.

According to all the above mentioned analysis the antibiotic was characterized as 2-(p-methoxybenzyl)-3,4-pyrrolidinediol-3-acetate (Fig. 1), on the basis of spectral data. The antibiotic was similar or identical to anisomycin (Umezawa, 1967). In this study, one antibacterial compound was purified from the culture broth of *S. sioyaensis* and identified as anisomycin. This compound was responsible for inhibition of *E. carotovora* growth *in vitro*. It may play an important role in disease suppression.

In conclusion, using chromatographic, mass spectrometer and other chemical analytical techniques, the authors isolated, purified and identified one antibacterial compound from the culture broth of *S. sioyaensis* that can inhibit *E. carotovora* growth *in vitro*. The antibiotic was identified as anisomycin and it may play an important role in soft rot disease suppression *in vivo*. Further investigations are necessary to determine the role of antibiotic in disease suppression.

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