J. Microbiol. Biotechnol. (2000), **10**(4), 522–528

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Antiviral Activity of Antibiotic Peptaibols, Chrysospemins B and D, Produced by *Apiocrea* sp. 14T against TMV Infection

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Received: July 20, 1999
Accepted: June 16, 2000

Abstract A total of about 300 fungal isolates from forest habitats were screened for inhibitors of tobacco mosaic virus (TMV) infection using its local lesion host, *Nicotiana tabacum* cv. Xanthi nc. One of the isolates, 14T, showed a strong activity against TMV infection, and was identified as an *Apiocrea* sp. based on its morphological characteristics. Rice was an optimum culture medium for its fermentation, and two antiviral compounds, KGT 141 and KGT 142, were resolved from the rice culture through column chromatography, TLC, and HPLC. By NMR and FAB-MS, the two compounds were identified as chrysospermins B (KGT 141) and D (KGT 142), both of which are peptaibols with 19-mer amino acids possessing an acetylated N-terminus and a hydroxy-amino acid (tryptophanol) at the C-terminus. Both compounds showed inhibitory activities against TMV infection, but chrysospermin D showed the stronger activity than chrysospermin B. The former exhibited 96.3% of TMV-inhibitory efficacy at the concentration of 100 μg/ml and 54.7% at 10 μg/ml, respectively. Furthermore, the chrysospermins were highly cytotoxic toward cancer cell lines of PC-3 (prostrate) and K562 (leukemia), and inhibited growth of the Gram-positive bacteria tested, especially the plant pathogenic bacterium *Corynebacterium lilium*. To the best of our knowledge, this is the first report on the inhibition of plant virus infection by antimicrobial peptaibols.

Key words: *Apiocrea* sp., chrysospermins, cytotoxic, peptaibol antibiotics, TMV-inhibitory

Inhibitors against plant virus infection have been identified from various microorganisms including bacteria, yeast, and fungi, and effective anti-phytoviral materials are mostly polysaccharides instead of proteins that are common effective components of plant extracts [14]. These microbial polysaccharides offer little potential for the development of agricultural viricides, because their mass production and usability are highly limited. Therefore, anti-phytoviral materials other than the macromolecules are necessary for the development of more useful and potent substances that can be applied either directly for the control of plant viruses or indirectly for molecular breeding of plants.

Antibiotics produced by actinomycetes are known to be mostly antimicrobial and some of them, such as blastidin S [15], actinomycin D [17], and antimycin A1 [29, 30], also have anti-phytoviral activities. Although actinomycetes have been a continual source of new metabolites, their production of antibiotics has already been fully scrutinized, so it has become extremely difficult to find materials with new physiological activities from them. On the other hand, fungi can still give more chances for the development of such useful materials because of their great diversity in nature.

Based on their actions, anti-phytoviral agents can be divided into two types of inhibitors: 1) anti-viral infection and 2) anti-viral multiplication [14], against which tobacco mosaic virus (TMV), one of the representative plant viruses, has been commonly tested using local lesion hosts such as *Nicotiana tabacum* cvs. Xanthi nc and Samson NN, *N. glutinosa*, and *Chenopodium amaranticolor*. Inhibitors against virus infection reduce the number of local lesions, while the virus multiplication-inhibitory substances usually reduce the size as well as the number of local lesions since they act on virus synthesis in plant cells: it is necessary for them to be introduced into plant tissues. The above TMV assay method is rapid and simple, and its result can be easily quantified by examining either of the two antiviral actions. This assay method to search physiologically active compounds may provide unexpected opportunities to find new compounds which were overlooked in the common *in vitro* antimicrobial assay systems.

We screened about 300 isolates obtained from forest habitats for new useful anti-phytoviral compounds by

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using the assay method. About 5% of the isolates appeared to produce inhibitors of TMV infection, one of which was isolate 14T, identified as *Apiocrea* sp. with the strongest and consistent antiviral activity. Two compounds were purified from the fungal culture of the isolate and they were found to be antimicrobial and cytotoxic to some cancer cells in addition to the inhibitory efficacy against TMV.

MATERIALS AND METHODS

Screening of Anti-Phytoviral Fungi

Mushrooms, wood-decaying fungi, puffballs, diseased leaves and branches, and rotten roots were collected from various forest habitats, including mountain areas of Kyeryong, Halla, Songni, and Deogyu, and their vicinities in Korea. Small pieces of the samples were plated on acidified potato dextrose agar (PDA) after surface-sterilizing with 1% sodium hypochlorite solution for 1 min. About 500 isolates were obtained from the surveyed areas, among which approximately 300 were tested for anti-phytoviral activity. The anti-phytoviral activity was assayed by the local lesion assay of TMV infection using the ethanol extracts of the fungal cultures. Briefly, fully grown fungal cultures on PDA were macerated in 5-fold volumes of 100% ethanol, centrifuged at 3,000 rpm for 15 min to remove fungal debris and agar, and dehydrated overnight in a desiccator *in vacuo*. The ethanol extract was dissolved in a half volume of distilled water, and an equal volume of 200-fold diluted TMV-infected plant sap (systemically infected *N. tabacum* cv. NC 82) was added. The mixture of culture filtrate and infected plant sap was inoculated on the half leaf of the local lesion host plant, *N. tabacum* cv. Xanthi nc, and the other half leaf was inoculated with the infected plant sap alone. The inoculated plant was placed at 25–27°C in a greenhouse, and the number of lesions formed on a treated half leaf was measured and compared to that on the other control half leaf at 3–4 days after inoculation. Three replicates were used for each culture filtrate.

Identification of Isolate 14T

In the above screening experiment, about 5% of the isolates showed over 80% TMV-inhibitory efficacies. Among them, isolate 14T from the surface of a bolete mushroom collected in Kyeryong mountain showed the strongest and most consistent antiviral effect in several antiviral efficacy trials. The morphological characteristics of the fungal isolate were examined for its identification using a scanning electron microscope (DSM 960A, Zeiss, Germany) and a light microscope (Axiophot, Zeiss, Germany). For electron microscopy, 7- to 10-day-old fungal cultures grown on PDA were fixed in Karnovksy's fixative with 0.01 M cacodylate buffer for 2 h, and postfixed with 1% osmium tetroxide in the same buffer. The fixed specimens were dehydrated in ethanol series, dried in a critical point dryer, and sputter-coated with gold before examining under the electron microscope. The same fresh fungal cultures were also examined under the light microscope.

Fermentation of Isolate 14T

Rice medium was used for the fermentation of the fungal isolate. Small pieces of 10-day-old plate cultures of isolate 14T grown on PDA were used to inoculate about 150 ml of boiled rice medium (60 g of rice in 80 ml distilled water) in 500-ml flasks, and incubated for 25 days at 27°C in an incubation chamber. The rice medium was mostly occupied by the fungus at around 15–20 days after inoculation, coloring to yellow or brownish yellow. The 25-day-old fungal cultures were used for the isolation of TMV-inhibitory compounds.

Isolation of TMV-Inhibitory Compounds

The isolation procedures are shown in Fig. 1. The rice culture of isolate 14T was extracted twice with 5-fold volumes of ethanol, and the residue of evaporated ethanol extract was separated using dual solvent systems (*n*-hexane-water, chloroform-water of the prior water extract, and *n*-butanol-water of the second water extract) while monitoring TMV-inhibitory activity. The water layer → water layer → *n*-butanol layer was TMV inhibitory. The residue of evaporated *n*-butanol was dissolved in a small volume of chloroform and subjected to column chromatography (25×400 mm) on

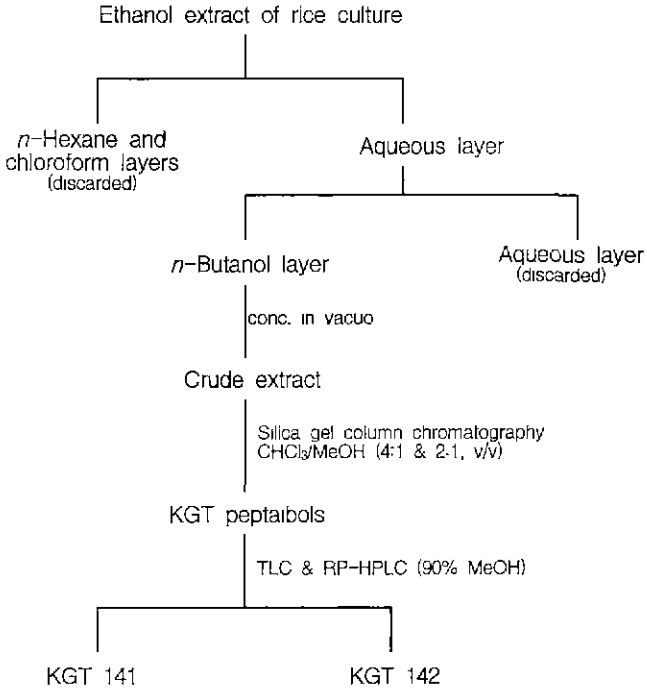

Fig. 1. Isolation procedures of KGT 141 and KGT 142 from rice culture of *Apiocrea* sp. 14T.

Silica gel 60 (0.063–0.2 mm, Merck, Darmstadt, Germany) using stepwise $CHCl_3$ and $CHCl_3$-MeOH (30:1 → 10:1 → 6:1 → 4:1 → 2:1 → 1:1, v/v). The fractions showing TMV-inhibitory activity in the eluents of $CHCl_3$-MeOH (4:1 and 2:1, v/v) were combined and subjected to ODS TLC using 90% methanol. Two main fractions with TMV inhibition were noted and separately collected using preparative TLC (Silica gel RP-18 F_{254S}, Merck, Darmstadt, Germany) with the same solvent system. Each fraction was concentrated and purified by preparative recycling HPLC on a 20 mm i.d. × 250 mm Nucleosil C18, 7 μm column in a 90% methanol solvent system, obtaining two main compounds, KGT 141 and KGT 142.

Physico-Chemical Properties and Molecular Structure of KGT 141 and KGT 142

The two purified compounds were subjected to instrumental analyses for their physico-chemical properties and structures. UV-visible and IR spectra were measured with a Shimazu UV-260 spectrophotometer and an Analect fx-6160 FT-IR spectrophotometer in KBr, respectively. Melting points were measured with a Fisher Johns melting point apparatus, and FAB- and HRFAB-MS were measured on a JEOL HX 100 mass spectrometer. The various spectra in MeOD were obtained on a Bruker NMR spectrometer at 400 MHz. For the structural elucidation, data on NMR spectra and ion FAB-MS were used.

Inhibitory Effect of KGT 141 and KGT 142 on TMV Infection

Each purified compound was dissolved in a small volume of ethanol to make a 2,000-μg/ml concentration. This solution was subjected to 10-fold serial dilutions with 10% ethanol, and was mixed with equal volumes of TMV-infected plant sap in 0.02 M phosphate buffer (pH 7.3), which was a 100-fold dilution of dried leaf debris. These mixtures were inoculated on the half leaves of the local lesion host plants, *N. tabacum* cv. Xanthi nc, and the other half leaves were inoculated with the control infected plant sap (TMV inoculum in 5% ethanol) as done in the screening experiment. The inoculated plants were placed at 25–27°C in the greenhouse, and the number of lesions formed on a treated half leaf was measured and compared to that on the other control half leaf 3–4 days after inoculation.

Antimicrobial Activity of KGT 141 and KGT 142 against Bacteria and Fungi

Antimicrobial activity was determined by the paper disc method using Mueller-Hinton agar and PDA for bacteria and fungi, respectively. Paper discs of 8 mm in diameter were soaked with the ethanol solution of each compound (30 μg per paper disc), and were placed on the media seeded with bacteria or fungi. One to two days later, clear zones around the paper discs were examined and their sizes were measured.

Cytotoxicity of KGT 141 and KGT 142 on Tumor Cells

Cytotoxicity of the purified compounds against human cancer cell lines was tested by the SRB method [25]. Cells were cultured in RPMI 1640 media with 10% fetal calf serum (FCS) containing the compound (dissolved in DMSO to give a final concentration of 0.1% DMSO) in wells of tissue culture microplates at 37°C in a 5% CO_2 incubator. The results are expressed as 50% effective dosage (ED_{50}) values which are concentrations of the compounds to inhibit cell growth by 50%.

RESULTS

Identification of Isolate 14T

The fungal isolate parasitized a bolete mushroom, and formed yellow and brownish yellow powdery spores on its gill and stipe. When the isolate was cultured on PDA, whitish mycelium initially formed but the culture turned yellow, because of the formation of yellow powdery spores. It grew well on PDA and malt extract agar, but poorly on Czapek dox agar or low nutritional medium like diluted PDA. It formed two types of spores, conidia and phialospores, on PDA (Fig. 2).

The width of aerial mycelium was 1.9–3.2 μm (2.4 μm in average). Conidia were round, had a coarse warty surface, was 11.5–13.8 μm (12.8 μm) in diameter, and formed perpendicularly to conidiophores of 8.9–22.2 μm (15.1 μm) in length. Phialospores were long, ellipsoidal, and 7.4–12.6× 2.6–4.0 μm (10.7×3.4 μm) in size. These morphological

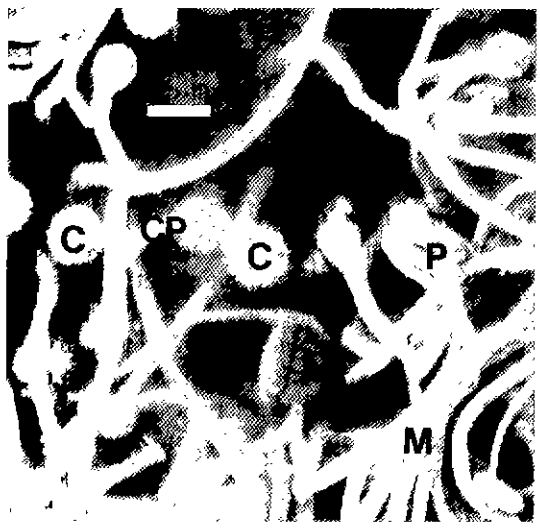

Fig. 2. Scanning electron micrograph of *Apiocrea* sp. 14T, showing aerial mycelium (M), conidiophores (CP), warty-surfaced conidia (C), and phialospores (P). Bar: 10 μm.

characteristics are very similar to those of *Sepedonium chrysospermum* [1] for, the teleomorph of which is *Apiocrea chrysosperma* except for some measurements of spores. Therefore, the fungal isolate was identified as an *Apiocrea* sp. and named *Apiocrea* sp. 14T. The teleomorph was not observed in cultivation. It was deposited at the Korea Research Institute of Bioscience and Biotechnology culture bank with the accession No. KCTC 8921P.

Production and Isolation of Peptaibol Antibiotics Inhibitory to TMV Infection

In the screening of fungal isolates for inhibition of TMV infection, the ethanol extract of the PDA culture of *Apiocrea* sp. 14T showed a very strong inhibitory effect against TMV infection, producing very few or sometimes no local lesions on the treated half leaves (Fig. 3). Initially, potato dextrose broth was used for the production of the active compounds, however, their productivity was relatively low, and the fungus grew poorly on other solid media like wheat and barley grains. On the other hand, the fungus grew well on rice medium, and produced anti-phytoviral materials readily.

As shown in Fig. 1, in the isolation procedures of the antibiotics from the rice culture of *Apiocrea* sp 14T, the TMV-inhibitory effect was observed in the *n*-butanol layer, but not in the water layer, of the previous water-soluble aliquot, and mostly in ethanol:water=5:5 and less in ethanol: water=7:3 on silica gel (ODS) column chromatography of the *n*-butanol extract (data not shown). The TMV-inhibitory materials were separated into chloroform:methanol=4:1 and 2:1 by silica gel column chromatography, condensed *in vacuo* and applied again to silica gel (ODS) column chromatography, in which the TMV-inhibitory materials were eluted in 90% methanol. These materials were separated by thin layer chromatography (TLC) in 90% methanol, and 2 main fractions were obtained. Each fraction was applied to HPLC for further purification. Finally, two purified TMV-inhibitory compounds were obtained and named KGT 141 and KGT 142. The average yield of KGT 141 and KGT 142 was approximately 10 mg/l and 30 mg/l of rice culture, respectively.

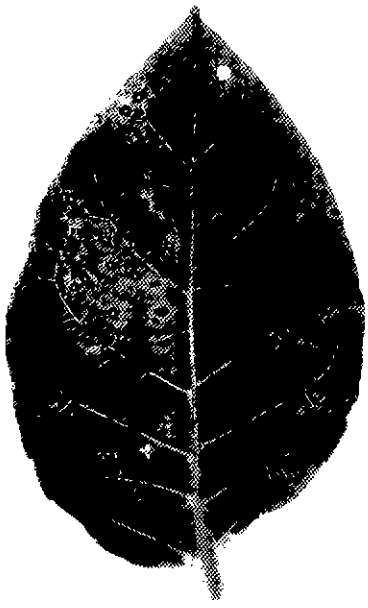

Fig. 3. Bioassay of TMV infection using a local lesion host. *Nicotiana tabacum* cv. Xanthi nc.
The left half leaf was inoculated only with TMV-infected plant sap, while the right half leaf was inoculated with the mixture of the culture extract and TMV-infected plant sap.

Physico-Chemical Properties and Molecular Structure of KGT 141 and KGT 142

With the help of basic and instrumental analyses of the chemicals purified through HPLC, the physico-chemical characteristics of both KGT 141 and KGT 142 were found to be almost identical. They are white powders soluble in DMSO and MeOH but not in water and *n*-hexane. Melting points for the compounds were over 250°C. The UV absorption maxima in MeOH were 282 nm (0.27391), 224 nm (1.7055), and 214 nm (1.4985) for KGT 141 and 282 nm (0.22003), 224 nm (1.4329), and 214 nm (1.3301) for KGT 142. Based on FAB-MS spectrometry, their molecular weights were determined to be 1,911 and 1,925 for KGT 141 and KGT 142, respectively. The molecular formulae of KGT 141 and KGT 142 were assigned as $C_{91}H_{142}N_{22}O_{23}$ and $C_{92}H_{142}N_{22}O_{23}$ by the HRFAB-MS. As revealed by MS and NMR analyses, they were peptides of 19 mers possessing a C-terminal tryptophanol (Trpol) and an acetylated N-terminus with rich non-standard amino acid residues such as aminoisobutyric acid (Aib). The respective molecular structures for KGT 141 and KGT 142 were (Ac-Phe- Aib- Ser- Aib- Aib- Leu- Gln- Gly- Aib- Aib- Ala- Ala- Aib- Pro- Iva- Aib- Aib- Gln- Trpol), and (Ac- Phe- Aib- Ser- Aib- Iva- Leu- Gln- Gly- Aib- Aib- Ala- Ala- Aib- Pro- Iva- Aib- Aib- Gln- Trpol) (Ac: acetyl, Phe: phenylalanine, Aib: aminoisobutyric acid, Iva: isovaline, Leu: leucine, Gln: glutamine, Gly: glycine, Ala: alanine, Pro: proline, Trpol: tryptophanol). These compounds are peptaibol antibiotics usually referred to as antibiotic peptides with typically 15 to 20 amino acid residues, non-standard amino acids like aminoisobutyric acid (Aib), and an alkyl N-terminus (usually acetyl) and a hydroxy-amino acid at the C-terminus. The two compounds matched well to chrysospermins B (KGT 141) and D (KGT 142) reported in a previous study [8].

Inhibitory Effect of Chrysospermins B (KGT 141) and D (KGT 142) on TMV Infection

In the local lesion assay using tobacco plants (*N. tabacum* cv. Xanthi nc), the purified antibiotics chrysospermins B and D showed strong inhibitory effects against TMV infection. Chrysospermin D showed a stronger inhibitory effect than

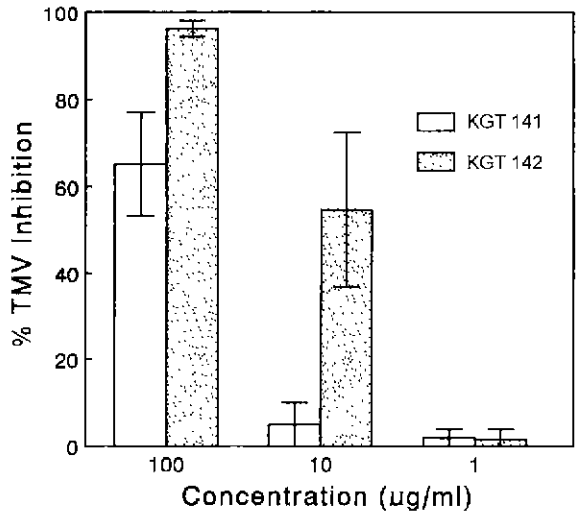

Fig. 4. Inhibitory effect of antibiotic materials produced by *Apiocrea* sp 14T on TMV infection.
The number of local lesions produced on one half leaf of *Nicotiana tabacum* cv. Xanthi nc was compared to that of the other half leaf at 3 days after TMV inoculation. Vertical bars represent standard deviations of three replications. KGT 141=Chrysospermin B, KGT 142=Chrysospermin D.

chrysospermin B, having 96.3% at the concentration of 100 μg/ml and 54.7% at 10 μg/ml (Fig. 4). However, the size of lesions was independent of the concentrations of the peptaibols and controls.

Antimicrobial Efficacy of Chrysospermins B and D

Chrysospermins B and D had similar antifungal and antibacterial activities (Table 1). They inhibited the growth of gram-positive bacteria, except for *Streptococcus* sp.,

Table 1. Antifungal and antibacterial activity of antibiotics produced by *Apiocrea* sp. 14T[a].

Test microorganism	Diameters of inhibition zone (mm)	
	Chrysospermin B	Chrysospermin D
Bacillus subtilis	10	10
Staphylococcus aureus R-209	11	10
Escherichia coli	13	10
Streptococcus sp.	9	0
Corynebacterium lilium	22	21
Candida albicans	0	0
Aspergillus niger	0	0
Magnaporthe grisea	0	0
Colletotrichum lagenarium	0	0
Fusarium solani	0	0
Mucor ramannianus	0	9
Alternaria mali	0	0
Saccharomyces cerevisiae	9	9

[a]A paper disc (8 mm in diameter) was treated with 30 μg of each purified compound.

Table 2. Cytotoxicity of antibiotics produced by *Apiocrea* sp. 14T on human cancer cell lines[a].

Cell line[b]	ED_{50} (μg/ml)[c]		
	Chrysospermin B	Chrysospermin D	Adriamycin
PC-3	4.11	1.71	1.00
A549	8.89	3.40	0.62
UACC62	6.12	2.01	0.26
EL4	16.14	10.28	0.45
K562	3.20	1.09	0.58

[a]Cells were cultured in RPMI 1640 media with 10% fetal calf serum (FCS) in a CO_2 incubator at 37°C.
[b]PC-3: prostrate cell line. A549 lung cancer cell line; UACC62: melanoma cell line EL4 lymphoma cell line: K562: leukemia cell line
[c]ED_{50}: calculated by quantal dose-response in probit analysis.

and were most effective on a plant-pathogenic bacterium *Corynebacterium lilium*. However, they had no significant inhibitory effect on all of the gram-negative bacteria and most of the fungi tested, except for weak antibiotic activities on *Mucor ramannianus* by chrysospermin D and on *Saccharomyces cerevisiae* by both.

Cytotoxicity of Chrysospermins B and D on Human Cancer Cells

Chrysospermins B and D had strong cytotoxic effects on human cancer cell lines (Table 2). Overall, chrysospermin D showed more cytotoxic activity than chrysospermin B, and the former exhibited the strongest growth inhibitory effect on PC-3 (prostrate) and K562 (leukemia), with ED_{50} of about 50% of a commercial anticancer agent, adriamycin, and the lowest against EL4 (lymphoma).

Discussion

The two compounds isolated from the rice culture of *Apiocrea* sp. 14T in our present study were identical to the chemicals described previously by Dornberger *et al.* [8]: The peptaibol antibiotics, chrysospermins B and D, produced by *A. chrysosperma* AP 101, correspond to our KGT 141 and KGT 142, respectively. In their study, two more peptaibol antibiotics, chrysospermins A and C, were reported, which had identical retention times and similar peak heights to chrysospermins B and D in the HPLC. In our study, two additional peptaibol compounds were also noted, however, based on the retention times in the HPLC and relative yields, they may be different from chrysospermins A and C [unpublished data]. Dornberger *et al.* [7] isolated the antibiotic peptides from the liquid culture of the fungus, while ours were from the fungal culture grown on rice medium.

It may be a phenomenon that different quantities of a specific substance are biosynthesized in different culture conditions, however different kinds of biosynthetic products may reflect the expression of different genes. In this respect,

Apiocrea sp. 14T may be genetically different from *A. chrysosperma* AP 101 because of different peptaibols production, but not at the species level since they are fungal parasites with almost identical morphological characteristics [8]. AP 104 is very closely related to 14T. Isolate 14T would be the same species as AP 101, differing only at the ecotype or subspecies level. However, a similar species, *Sepedonium ampullosporum*, produces ampullosporin, a 15-membered peptaibol [23], but *Trichoderma harzianum* produces trichozianins [4], 19-mers similar to chrysospermins. This indicates that taxonomic similarities between producing species may not be exclusively related to the types of peptaibols.

Antimicrobial peptides with potent antimicrobial activities against bacteria, protozoa, fungi, and viruses have been identified from various sources [11, 18, 19]. The mechanism of their activity is to form multimetric pores directly through the lipid bilayer of the cell membrane [20, 27]. In particular, peptaibols invariably exhibit antimicrobial activities by forming the pores (ion channels) through membranes [2, 5, 9, 12, 21, 24], while other types of antibiotics (polythiazoles) containing peptide have different modes of action, such as the inhibition of fungal cell wall synthesis [28]. The pores so formed lead to the loss of osmotic balance and cell death. Antibiotic peptaibols have been shown to exhibit various biological activities, including being hemolytic or inhibitory to platelet aggregation [6, 7], neuroleptic [23], antiamoebic [26], as well as being antibacterial and antifungal [3, 4, 8]. However, no anti-phytoviral effect of antibiotic peptaibols has ever been reported.

In our study, a new biological activity of peptaibol antibiotics, the inhibition of plant virus infection, would be added to the above described activities. In particular, chrysospermin D almost completely inhibited local lesion formation by TMV infection on the tobacco leaves at 100 μg/ml in replicated tests. In the most extreme case, the local lesion formation was reduced by 94.4% at 10 μg/ml, and even by 83.3% at 1 μg/ml as well [unpublished data]. However, lesion size was not affected by chrysospermin D, and local lesions were readily formed on the tobacco leaves when the compound was applied 2 and more hours after TMV inoculation, indicating that it may not be introduced into the plant tissues to exhibit systemic activity. These considerations suggest that the compound may inactivate the virus infectivity *in vitro* or inhibit virus infection, but not virus multiplication. The inhibition of the virus infection may be due to the formation of a virus-inhibitor complex rather than by blocking the host receptor sites, since the TMV-inhibitory efficacy was rapidly diminished with time when TMV was inoculated on leaves after the chemical treatment (unpublished data). This indicates that the TMV-inhibitory activity may be modulated by mechanisms other than membrane channel formation. Further study is required to clarify specific actions of the antiviral agents.

Rice seemed to be a good nutritional source for the growth of *Apiocrea* sp. 14T and the production of the antiviral peptaibols. Their mass production and widespread application, however, are still very limited because of the slow fungal growth. Chemical synthesis of peptaibols and their analogues is an alternative, but is not cost-effective for production of the peptaibols with more than 10 amino acid residues. With non-peptaibol polypeptides like moricin and magainin, bacterial or modified bacterial systems through genetic engineering have been utilized for more cost-effective production of the peptides [12, 15]. However, it is hardly applicable for peptaibols because of their modified N- and C-terminal residues and rich non-standard amino acids. Furthermore, chrysospermins B and D had no TMV-inhibitory effecsts in systemic tobacco plants such as NC 82 tobacco, a commercial tobacco cultivar in Korea. Therefore, the use of the peptaibol antibiotics as agricultural viricides *in situ* appears to be very unlikely.

For antimicrobial and cytotoxic effects of chrysospermins B and D, however, our study may give some prospective results. They showed antimicrobial activity, most highly against *C. lilium*, a gram-positive plant pathogenic bacterium. The peptaibol antibiotics, especially chrysospermin D, also showed a strong cytotoxic effect on some cancerous cells tested. They can be used as therapeutic chemicals for human cancers and as bactericides. In cytotoxic effects to human cancer cells, chrysospermin D invariably showed stronger activities than chrysospermin B. They differ structurally in only one amino acid residue; aminoisobutyric acid for chrysospermin B and isovaline for chrysospermin D at the fifth position from the acetylated N-terminus. Since conformational changes occur by changes of amino acid and lead to different behavior of peptaibol compounds [9, 21], the difference of amino acid residue may affect the cytotoxic efficacies.

Acknowledgments

This study was supported by a grant (TA-411) from the Korea Tobacco and Ginseng Corporation.

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