Anti-plant viral activity of peptaibols, trichorzins HA II, HA V, and HA VI, isolated from Trichoderma harzianum HK-61

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Three peptaibols, trichorzins HA II (1), HA V (2), and HA VI (3), were isolated from okara fermented with Trichoderma harzianum HK-61 as anti-plant viral agents. Their structures were confirmed by spectroscopic and chemical methods. At micro molar concentrations, the trichorzins inhibited infections by Cucumber mosaic virus in the cowpea plant Vigna sesquipedalis. © Pesticide Science Society of Japan

Keywords: Cucumber mosaic virus, Trichoderma harzianum, anti-plant viral activity, peptaibols, trichorzins.

Introduction
Viruses are among the pathogens most agriculturally detrimental to crops. Plant viral diseases cause serious economic losses in agriculture by reducing yield and quality. Because of the simple form of viruses, which consist of a segment of DNA or RNA encoding the genes required for their own multiplication in hosts and coat proteins, the chemical control of their diseases remains difficult or impossible.1,2) Therefore, the discovery of compounds that can inhibit plant viral infections continues to be a priority.

In previous studies, we yielded several fungal strains from soil samples collected in Japan that produced unique natural products. For example, Penicillium simplicissimum ATCC 90288 produced insecticidal indole alkaloids, okaramines,3–8) and the unidentified ascomycete OK-128 produced paralytic cyclic peptides, PF1171s.9,10) In this study, we have screened the fermentation extracts of fungal strains to find anti-plant viral compounds. Here, we report the isolation, identification, and anti-plant viral activity of three peptaibols—trichorzins HA II (1), HA V (2), and HA VI (3) (Fig. 1)—produced by Trichoderma harzianum HK-61.

Materials and Methods

1. Fermentation
T. harzianum HK-61 was isolated from a soil sample collected in Sakai (Japan) in the usual manner. Identification of this strain was carried out at the CBS Fungal Biodiversity Centre (The Netherlands). A loopful of spores from a slant culture of the strain was inoculated into 30 g of okara in a Petri dish 9 cm in diameter, and cultivation was carried out at 25°C for 14 days.

2. Extraction and isolation
The okara (2 kg) that had been fermented with strain HK-61 was soaked in MeOH for 2 days. Evaporation of the MeOH gave an aqueous concentrate, which was extracted with EtOAc. The EtOAc extract was concentrated and subsequently chromatographed on Wakogel C-200 (Wako Pure Chemical) by eluting with n-hexane and an increasing ratio of EtOAc to afford active eluates (5.0 g; 60 and 80% EtOAc). The active eluates were further chromatographed on Chromatorex ODS (Fuji Silysia Chemical) by eluting with H2O and an increasing ratio of MeOH to afford an active eluate (1.5 g; 100% MeOH). The active fraction was subjected to preparative HPLC [column, Inertsil ODS-3 10×250 mm (GL Sciences); solvent, 75% MeOH in 0.05% aq. TFA; flow rate, 4 mL/min, 50 times] to yield 1 (90 mg), 2 (320 mg), and 3 (150 mg).

3. Spectroscopic analysis of trichorzin HA V (2) and its partial acid hydrolysates
NMR experiments were carried out in DMSO- d6 using a JNM AL-400 NMR spectrometer (JEOL). Chemical shifts were referenced to the solvent peak (δH 2.49, δC 39.7) as an internal standard. FAB-MS was recorded on a JMS-700 (JEOL) using glycerol as the matrix. The 1H- and 13C-NMR signals of 2 could not be assigned due to the severe overlapping of signals. FAB-MS

Fig. 1. Structures of trichorzins HA II (1), HA V (2), and HA VI (3).
data for 2 are shown in Fig. 2. Compound 2 was partially hydrolyzed in 6 M HCl for 90 min at 90°C to afford five fragment peptides, 4–8. The structures of the peptides were determined by 1D-NMR (1H, 13C) and 2D-NMR (1H–1H COSY, HMOC, HMBQC, HMBC). Spectroscopic data for 4: 1H-NMR (400 MHz) δ: 0.71 (3H, dd, J = 7.3, 7.7 Hz, Iva-γ), 0.83 (3H, d, J = 6.7 Hz, Leu-δ), 0.90 (3H, d, J = 6.7 Hz, Leu-δ), 1.34 (3H, s, Aib–β), 1.35 (3H, s, Aib–β), 1.37 (3H, s, Iva–β), 1.49 (2H, m, Leu–β), 1.63 (1H, m, Leu–γ), 1.74 (1H, m, Iva–β), 1.84 (2H, m, Pro–β), 1.89 (1H, m, Pro–β), 2.01 (1H, m, Iva–β), 2.28 (1H, m, Pro–β), 3.26 (2H, br, Pro–δ), 4.20 (1H, br, Pro–α), 4.36 (1H, m, Leu–α), 7.13 (1H, s, Iva–NH), 8.16 (1H, s, Aib–NH), 8.54 (1H, d, J = 7.9 Hz, Leu–NH), 9.20 (1H, br, Pro–NH). 13C-NMR (100 MHz) δ: 7.9 (Iva–γ), 21.3 (Iva–δ), 21.9 (Iva–δ), 22.8 (Iva–β), 23.1 (Pro–γ), 24.0 (Leu–γ), 24.5 (Aib–β), 24.8 (Aib–β), 28.5 (Iva–β), 29.4 (Pro–β), 40.5 (Leu–δ), 45.7 (Pro–δ), 51.6 (Leu–α), 56.2 (Aib–α), 58.7 (Pro–α), 59.0 (Iva–α), 167.8 (Pro–C=O), 171.0 (Leu–C=O), 172.5 (Aib–C=O), 175.1 (Iva–C=O). FAB-MS: m/z 413 [M+H]+ (C73H29N13O13). Spectroscopic data for 5: 1H-NMR (400 MHz) δ: 0.74 (3H, t, J = 7.5 Hz, Iva–γ), 1.36 (3H, d, J = 8.5 Hz, Ala–β), 1.38 (3H, s, Iva–β), 1.39 (3H, s, Iva–β), 1.44 (3H, s, Aib–β), 1.76 (1H, m, Iva–β), 2.03 (1H, m, Iva–β), 2.36 (2H, br, Ala–α), 7.15 (1H, s, Iva–NH), 8.00 (2H, br, Ala–NH), 8.35 (1H, s, Aib–NH). 13C-NMR (100 MHz) δ: 7.9 (Iva–γ), 17.0 (Ala–β), 21.8 (Iva–β), 24.0 (Aib–β), 25.3 (Aib–β), 28.6 (Iva–β), 48.2 (Ala–α), 56.5 (Aib–α), 59.1 (Iva–α), 168.6 (Ala–C=O), 171.7 (Aib–C=O), 174.9 (Iva–C=O). FAB-MS: m/z 274 [M+H]+ (C12H22N4O5). Spectroscopic data for 6: 1H-NMR (400 MHz) δ: 0.90 (3H, d, J = 6.1 Hz, Leu–δ), 0.91 (3H, d, J = 6.1 Hz, Leu–δ), 1.38 (3H, s, Aib–β), 1.42 (3H, s, Aib–β), 1.51 (1H, m, Leu–γ), 1.59 (1H, m, Leu–γ), 1.70 (1H, m, Leu–γ), 3.72 (1H, br, Leu–α), 8.02 (2H, br, Leu–NH), 8.49 (1H, s, Aib–NH). 13C-NMR (100 MHz) δ: 21.7 (Leu–δ), 22.4 (Leu–δ), 23.2 (Leu–δ), 24.4 (Aib–β), 24.7 (Aib–β), 40.0 (Leu–β), 50.6 (Leu–α), 55.2 (Aib–α), 167.7 (Leu–C=O), 174.3 (Aib–C=O). FAB-MS: m/z 217 [M+H]+ (C10H22N3O5). Spectroscopic data for 7: 1H-NMR (400 MHz) δ: 0.82 (3H, d, J = 6.8 Hz, Val–γ), 0.89 (3H, d, J = 6.8 Hz, Val–γ), 1.35 (3H, s, Aib–β), 1.36 (3H, s, Aib–β), 1.43 (3H, s, Aib–β), 1.43 (3H, s, Aib–β), 1.97 (2H, m, Glu–β), 2.00 (1H, m, Val–β), 2.36 (2H, t, J = 7.8 Hz, Glu–γ), 3.81 (1H, br, Glu–α), 4.13 (1H, dd, J = 7.3, 8.5 Hz, Val–α), 7.09 (1H, d, J = 8.8 Hz, Val–NH), 7.95 (1H, s, Aib–NH), 8.03 (2H, br, Glu–NH2), 8.37 (1H, s, Aib–NH). 13C-NMR (100 MHz) δ: 18.0 (Val–γ), 18.9 (Val–γ), 24.4 (Aib–β), 24.6 (Aib–β), 24.7 (Aib–β), 25.0 (Aib–β), 26.1 (Glu–γ), 28.9 (Glu–γ), 30.5 (Val–β), 51.7 (Glu–α), 54.7 (Aib–α), 56.6 (Aib–α), 57.5 (Val–α), 167.3 (Glu–C=O), 169.7 (Val–C=O), 172.3 (Aib–C=O), 172.8 (Glu–γ), 174.8 (Aib–C=O). FAB-MS: m/z 417 [M+H]+ (C10H16N4O5). Spectroscopic data for 8: 1H-NMR (400 MHz) δ: 0.74 (3H, t, J = 7.6 Hz, Iva–γ), 0.82 (6H, d, J = 6.7 Hz, Val–γ′), 1.26 (3H, s, Iva–β), 1.38–1.44 (15H, Aib1–β′, Aib2–β′, Aib3–β′), 1.40 (3H, s, Aib–β), 1.43 (3H, d, J = 7.0 Hz, Ala–β), 1.75 (1H, m, Iva–β), 2.03 (3H, Iva–β), 2.19 (1H, m, Val–β), 2.33 (2H, t, J = 7.5 Hz, Glu–γ), 3.58 (1H, dd, J = 5.8, 18.0 Hz, Gly–α), 3.76 (1H, d, J = 5.8, 18.0 Hz, Gly–α), 3.86 (1H, br, Ala–α), 3.96 (1H, m, Glu–α), 4.07 (1H, dd, J = 5.5, 8.9 Hz, Val–γ), 6.93 (1H, d, J = 8.9 Hz, Val–NH), 7.47 (1H, t, J = 5.8 Hz, Gly–NH), 7.49 (1H, s, Aib–NH), 7.66 (1H, d, J = 6.1 Hz, Glu–NH), 7.79 (1H, s, Aib–NH), 7.85 (1H, s, Iva–NH), 7.98 (2H, br, Ala–NH), 8.75 (1H, s, Aib–NH). 13C-NMR (100 MHz) δ: 7.3 (Iva–γ), 16.5 (Ala–β), 17.3 (Val–γ), 19.0 (Val–γ′), 22.4 (Iva–β′), 23.6–25.6 (Aib1–β′, Aib2–β′, Aib3–β′), 25.7 (Glu–γ), 25.9 (Aib–β′), 26.5 (Iva–β), 28.9 (Val–β′), 30.3 (Glu–γ′), 40.7 (Gly–α), 48.2 (Ala–α), 54.5 (Glu–α), 55.9 (Aib–α), 56.1 (Aib–α), 56.3 (Aib–α), 57.9 (Val–α), 59.0 (Iva–α), 169.4 (Ala–C=O), 170.0 (Val–C=O), 170.7 (Gly–C=O), 172.0 (Glu–C=O), 173.6 (Ala–C=O), 174.0 (Aib–C=O, Aib3–C=O), 175.8 (Iva–C=O). FAB-MS: m/z 729 [M+H]+ (C18H19N2O11).
detection, 340 nm) over 60 min. The retention times for Marfey’s derivatives of 2 were: l-Glu, 14.8 min; Gly, 16.2 min; l-Ala, 19.0 min; l-Pro, 19.5 min; Aib, 24.8 min; l-Val, 28.3 min; d-Iva, 31.5 min; l-Leuol, 32.8 min; l-Leu, 36.2 min. The retention times for Marfey’s derivatives of 1 were: l-Glu, 14.2 min; Gly, 15.5 min; l-Ala, 18.2 min; l-Pro, 18.6 min; Aib, 24.2 min; l-Val, 27.6 min; d-Iva, 30.7 min; l-Leuol, 32.8 min; l-Leu, 35.9 min. The retention times for Marfey’s derivatives of 3 were: l-Glu, 14.3 min; Gly, 15.7 min; l-Ala, 18.4 min; l-Pro, 18.9 min; Aib, 24.4 min; l-Val, 27.7 min; d-Iva, 30.8 min; l-Leuol, 32.8 min; l-Leu, 35.9 min.

6. Bioassay

An isolate of CMV propagated in tobacco (N. tabacum cv. Xanthi) was purified as described. MeOH extracts of okara (the insoluble residue of whole soybean) fermented with fungal strains were used for the bioassay. As a result of screening 200 strains, we found that T. harzianum HK-61 produces antiviral compounds against CMV; therefore, we isolated the active compounds. The MeOH extract of okara fermented with HK-61 was concentrated in vacuo and assigned the respective fragment ions derived from the cleavage of amide bonds (Fig. 2). Thus, we concluded that compound 2 was trichorzin HA V (Fig. 1), which was previously isolated from T. harzianum M-903602 as an antibacterial compound. Similarly, compounds 1 and 3 were identified as trichorznas HA II and HA VI (Fig. 1), respectively.

Finally, the antiviral activity of the purified trichorzins was examined. The compounds were added to hydroponic cultures of cowpea plants, and CMV was inoculated into the leaves. Compound 2 showed the strongest effects, 80.5% (5 μM) and 90.6% (10 μM) inhibition, against CMV (Table 1). Compounds 1 and 3 exhibited 42.6% and 68.5% inhibition at a concentration of 10 μM. This is the first report of the anti-plant viral activity of trichorzins.

Peptaibols are characterized by an N-terminal acylated amino acid residue and a C-terminal amino alcohol on a lipophilic chain that includes many a,a-dialkylated amino acids, such as Aib and Iva. Numerous peptaibols have been isolated from Trichoderma spp. and several other fungi mainly as antimicrobial substances. Previously, Yeo and co-workers isolated two peptaibols, peptaivirins A and B, from the unidentified fungus KGT142 as antiviral agents against infection by Tobacco mosaic virus (TMV) in the tobacco plant Nicotiana tabacum cv. Xanthi-nc. The 18mer peptaibols TvBI and TvBII from T. viriden Gv29-8 elicited defense responses in the cucumber plant Cucumis sativus that resulted in resistance against several bacteria. Furthermore, trichokonins isolated from T. pseudokoningii SMF2 were revealed to induce defense responses and systemic resistance in N. tabacum var. Samsun NN against TMV infections. These results suggest that trichorzins may also induce defense responses in cowpea plants and could cause resistance to CMV. Trichorzins’ mechanism of action against CMV in cowpea plants would be an interesting future topic of study.

Table 1. Inhibitory activity of trichorzins against the CMV infection

| Compound          | Concentration (μM) | Inhibition (%) a |
|-------------------|--------------------|-----------------|
| Trichorzin HA II  | 5                  | 33.5±5.3        |
|                   | 10                 | 42.6±2.7        |
| Trichorzin HA V   | 5                  | 80.5±4.6        |
|                   | 10                 | 90.6±4.2        |
| Trichorzin HA VI  | 5                  | 16.5±2.8        |
|                   | 10                 | 68.5±5.7        |

a Data shown are the mean±SE for three replicates.

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