Journal of Microbiology (2014) Vol. 52, No. 1, pp. 64–70
DOI 10.1007/s12275-014-3520-7

Cyclic Dipeptides from Lactic Acid Bacteria Inhibit the Proliferation of Pathogenic Fungi

Min-Kyu Kwak[1], Rui Liu[1], Min-Kyu Kim[1#], Dohyun Moon[2], Andrew HyoungJin Kim[1], Sung-Hyun Song[1], and Sa-Ouk Kang[1*]

[1]Laboratory of Biophysics, School of Biological Sciences, and Institute of Microbiology, Seoul National University, Seoul 151-747, Republic of Korea
[2]Beamline Division, X-ray Research II Team at Pohang Accelerator Laboratory, POSTECH, Pohang 790-784, Republic of Korea
[#]Present address: Marine Biotechnology Research Division, Korea Institute of Ocean Science and Technology, Ansan 426-744, Republic of Korea

(Received Oct 8, 2013 / Revised Nov 27, 2013 / Accepted Dec 2, 2013)

***Lactobacillus plantarum* LBP-K10 was identified to be the most potent antifungal strain from Korean traditional fermented vegetables. The culture filtrate of this strain showed remarkable antifungal activity against *Ganoderma boninense*. Five fractions from the culture filtrate were observed to have an inhibitory effect against *G. boninense*. Also, the electron ionization and chemical ionization indicated that these compounds might be cyclic dipeptides. Of the five active fractions, two fractions showed the most significant anti-*Ganoderma* activity, and one of these fractions inhibited the growth of *Candida albicans*. These compounds were identified to be *cis*-cyclo(L-Val-L-Pro) and *cis*-cyclo(L-Phe-L-Pro), as confirmed by X-ray crystallography.**

***Keywords*:** *Lactobacillus plantarum* LBP-K10, culture filtrate, *cis*-cyclo(L-Val-L-Pro), *cis*-cyclo(L-Phe-L-Pro), *Ganoderma boninense*, *Candida albicans*

Introduction

Lactic acid bacteria are widely considered to be natural antimicrobial tools that can be found in fermented substrates, such as many types of animal and plant materials (Holzapfel *et al.*, 2001). It has been suggested that this antagonistic property can be found in intestinal and food-borne pathogens (Gibson *et al.*, 1997). The preventive effects of lactic acid bacteria include the inhibition of adherence, establishment, replication, and various pathogenic actions (Saavedra, 1995). These bacteria have been reported to decrease pH by producing volatile short-chain fatty acids, such as lactic acid, acetic acid and propionic acid. The following functions have also been reported: reduction in the environmental redox potential, production of hydrogen peroxide under anaerobic conditions, excretion of small molecules that are toxic to pathogens, and synthesis of specific inhibitory compounds, such as bacteriocin-like peptides (Havenaar *et al.*, 1992).

The microbe-inhibiting substances produced by lactic acid bacteria have been postulated to be bacteriocin-like peptides, including bacteriocin, plantaricin, and pediocin. The identities of these inhibitors have been debated, but those particular compounds have demonstrated activity against Gram-positive and Gram-negative bacteria although their antimicrobial functions are not completely understood (Abee *et al.*, 1995). Additionally, small molecules, such as the secondary metabolites of lactic acid bacteria that are effective against microbes, have been investigated to demonstrate or determine their effects on cellular metabolism during growth and fermentation. These molecules include reutericyclin; 3-phenyllactic acid; benzoic acid; methylhydantoin; benzeneacetic acid; 2-propenyl ester; mevalonolactone; 2,6-diphenyl-piperidine; 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo [1,2-a;1',2'-d]pyrazine; and cyclic dipeptides as previously reported (Niku-Paavola *et al.*, 1999; Gänzle *et al.*, 2000; Ström *et al.*, 2002; Nardi *et al.*, 2005; Li *et al.*, 2012; Wang *et al.*, 2012).

Cyclo(Gly-Leu) from *Lb. plantarum* VTT E-78076 was shown to be an antifungal compound with activity against the plant fungal pathogen *Fusarium avenaceum* (*Gibberella avenacea*) VTT-D-80147 (Niku-Paavola *et al.*, 1999). Cyclo (Phe-Pro) and cyclo(Phe-trans-4-OH-Pro) from *Lactobacillus plantarum* MiLAB 393 have been investigated as antifungal substances (Ström *et al.*, 2002). *Lb. casei* AST18 isolated from spoilage cheese produces the antifungal compound cyclo(Leu-Pro), which is active against *Penicillium* sp. (Li *et al.*, 2012). Fungal inhibition by cyclic dipeptides has been reported, and the antifungal activity was not solely related to the production of organic acids (Magnusson *et al.*, 2003). Cyclo(Phe-Pro) and cyclo(Leu-Pro) from the *Streptomyces* species have been reported to inhibit pathogenic yeasts, including *Candida albicans* and *Cryptococcus neoformans* (Rhee, 2004). Cyclo(Trp-Trp) from *Streptomyces* sp. KH29 showed significant inhibitory activity against the multidrug-resistant *Acinetobacter baumannii* and also against other bacterial and fungal strains (Lee *et al.*, 2010). Cyclo(Phe-Pro), cyclo(Trp-Pro), cyclo(Trp-Trp), and histidine-containing cyclic dipeptides inhibited fungal strains such as *C. albicans*, *Aspergillus niger* and *Penicillium notatum* (Graz *et al.*, 1999; McCleland *et al.*, 2004). Research on cyclic dipeptides has focused on their structures, methods of synthesis, and their antimicrobial functions (Prasad, 1995; O'Neill and Blackwell, 2007; Trabocchi *et al.*, 2008; Budesinsky *et al.*, 2010). Furthermore, basal stem rot or upper stem rot by basidiomycete fungal

*For correspondence. E-mail: kangsaou@snu.ac.kr; Tel.: +82-2-880-6703; Fax: +82-2-888-4911

pathogens have killed various oil palm plants; in particular, the pathogen *Ganoderma boninense*, which causes stem rot disease, has been involved in many of these infections (Pilotti *et al*., 2004; Pilotti, 2005).

Our previous study isolated *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) from the culture filtrate of *Lb. plantarum* LBP-K10 and determined their structures; these compounds significantly inhibited the viral proliferation and infectivity of the influenza A virus (Kwak *et al*., 2013). Therefore, in the present study, we focused on cyclic dipeptides with antifungal activity against *G. boninense*. Our research will suggest new antifungal cyclic dipeptides that differ from the previously reported antibiotics, fungicides, and competitive microbial materials.

Materials and Methods

Strain

The isolation of lactic acid bacteria from three types of Korean fermented-plant sources and the propagation of identified isolates were described previously (Kwak *et al*., 2013). The *G. boninense* isolate (GMR3) (Rees *et al*., 2007) for growing mycelium was supplied by Cilic BioEngineering (CBE Sdn. Bhd., Malaysia). This isolate was grown on *Ganoderma* selective medium (GSM) and was maintained as described previously (Rees *et al*., 2007). *C. albicans* was grown on minimal defined SD medium as reported previously (Sherman, 2002).

Fractionation of antifungal substances

All procedures for the purification steps were performed according to the previously reported method (Kwak *et al*., 2013).

Antifungal test

All procedures for the antifungal activity of the culture filtrate from isolated lactic acid bacteria were performed according to the previously proposed with some modifications (Chong *et al*., 2009; Bivi *et al*., 2010). Each *Ganoderma* mycelium for the antifungal activity was incubated with the culture filtrate of lactic acid bacteria. After the incubation for 24 h, mycelium from *G. boninense* were loaded onto potato dextrose agar (PDA) plates using punctures that were 8.0 mm in diameter and cultivated for 7 days at 28°C.

Assays for the anti-*Ganoderma* activity of the fractionated substances from the culture filtrate were performed. Mycelium from *G. boninense* were loaded onto 6-well PDA plates (3.0 ml) using punctures that were 8.0 mm in diameter. After the seeding of *G. boninense* mycelium, 4.8–6.4 mg of lyophilized fractions were suspended in sterilized distilled water. Each fraction was loaded onto 6-well PDA plates and cultivated for 7 days at 28°C.

The anti-*Candida* activities of the fractions from the culture filtrate from *Lb. plantarum* LBP-K10 were investigated as follows. *Candida* cells were inoculated onto 6-well plates with dextrose minimal defined SD agar medium (3.0 ml) and cultured for 3–4 days at 28°C. The initial inoculation contained 1.0×10^4 *Candida* cells in each well.

The antifungal activity was calculated by comparing the diameter of the initial inoculates to the diameter of the mycelium or colony after it grew and spread.

Mass analysis

Gas chromatography-mass spectrometry (GC-MS) was used for the electron ionization (EI) and chemical ionization (CI) of the fractions as described previously (Kwak *et al*., 2013).

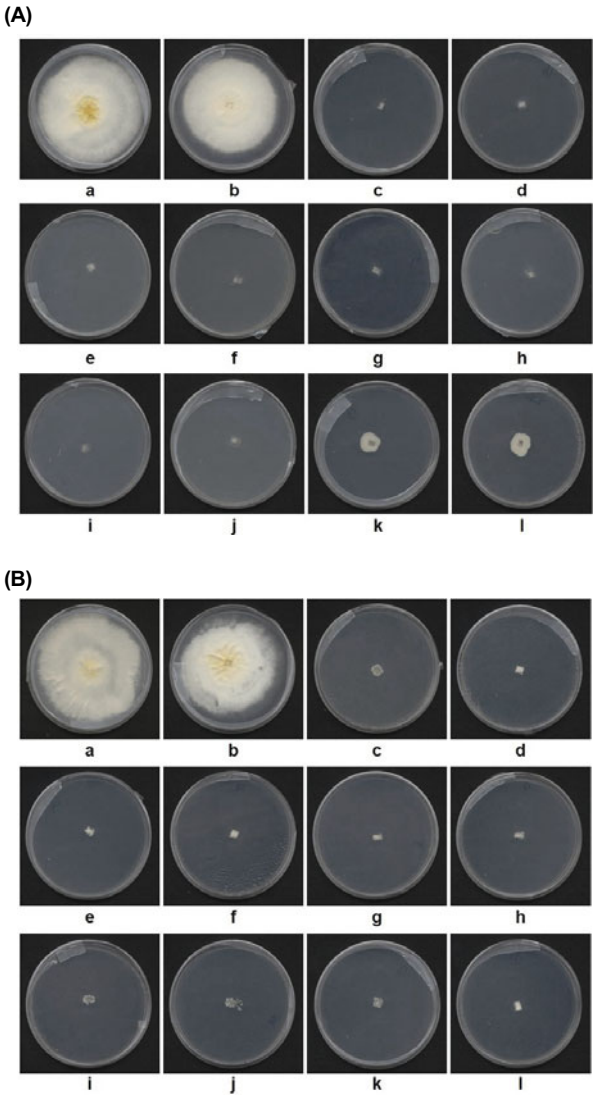

Fig. 1. Antifungal activity against *G. boninense* of the culture filtrate from various lactic acid bacteria. (A) Effects of heat-treated culture filtrate against *G. boninense*. *Ganoderma* mycelium were loaded onto a PDA plate and cultured for 7 days at 28°C. The following lactic acid bacterial strains were used: a, Reference; b, MRS; c, *Lb. sakei* LBP-B01; d, *Ln. kimchii* LBP-B02; e, *Lc. lactis* LBP-S03; f, *W. hellenica* LBP-S08; g, *Ln. citreum* LBP-K03; h, *Ln. mesenteroides* LBP-K06; i, *Lb. brevis* LBP-K09; j, *Lb. plantarum* LBP-K10; k, *Ln. pseudomesenteroides* LBP-K14 and l, *W. confusa* LBP-K16. (B) Effects of culture filtrate that were not treated with heat. All experimental procedures and the studied lactic acid bacterial strains are described above.

X-ray crystallographic analysis

The crystals of all fractions were coated with paratone-N oil, and the diffraction data were measured at 95 K with synchrotron radiation (λ = 0.66999 Å) on an ADSC Quantum-210 detector with a silicon (111) double crystal monochromator (DCM) at the 2D SMC beamline at the Pohang Accelerator Laboratory, Korea. ADSC Q210 ADX program 1 was used for data collection (detector distance is 62 mm, omega scan; $\Delta\omega = 1°$, exposure time is 20 sec per frame), and HKL3000sm (Ver. 703r) was used for cell refinement, data reduction and absorption corrections. The crystal structures of all fractions were solved by the direct method with the SHELXTL-XS program and were refined by full-matrix least-squares calculations with the SHELXTL-XL (Ver. 2008) program package. These crystal structures were deposited at the Cambridge Crystallographic Data Centre (https://www.ccdc.cam.ac.uk/services/structure_deposit/). The data have been assigned to the following deposition numbers: CCDC 937497 and CCDC 937534 for the 7th and 17th fractions, respectively.

Results

Identification of isolates from kimchi

Approximately 400 strains of lactic acid bacteria, including *Leuconostoc* spp., *Lactobacillus* spp., *Weissella* spp., and *Lactococcus lactis*, were isolated from three types of Korean kimchi which were prepared using various vegetables in our study as previously reported (Kwak *et al*., 2013). These strains were identified using 16S rDNA sequencing methods with PCR amplification (data not shown). Of these isolated bacterial strains, 205 isolates effective against microbes were selected and 30 of these isolates had a culture filtrate that preliminarily showed higher antimicrobial activity than that of the other isolates from the culture filtrate (data not shown).

Anti-*Ganoderma* effect of the culture filtrate

We investigated the anti-*Ganoderma* activity using heat-treated and non-heat-treated filtrate from three-day cultures after filtering the supernatants with a 0.22 μm-cellulose acetate membrane; nine types of isolates and most of these strains showed significant antifungal activity against *Ganoderma* mycelium (Fig. 1). This antifungal effect of the culture filtrate against *G. boninense* suggested that this activity against *G. boninense* could be attributed to a mixture of small molecules. Preliminary tests indicated that the majority of the compounds from the culture filtrate were effective against fungal pathogens. Of the isolated lactic acid bacterial strains, *Lb. plantarum* LBP-K10 had a culture filtrate that showed higher anti-*Ganoderma* activity than that of the other isolates from the culture filtrate. Therefore, *Lb. plantarum* LBP-K10 is mainly used for the further investigations of the antifungal compounds.

Fractionation of the culture filtrate

The antifungal substances from the filtrate of the three-day culture of *Lb. plantarum* LBP-K10 were fractionated, which yielded seventeen different fractions (designated F1-F17) (Kwak *et al*., 2013). After methylene chloride extraction of the culture filtrate, the fractions were collected from the chromatographic separation patterns; all resulting fractions were prepared as dehydrated powders. To identify the antifungal fractions, anti-*Ganoderma* and anti-*Candida* assays with the purified substances were performed after EI and CI structural determination using GC-MS (data not shown). To determine the molecular structures of these fractions, the EI and CI values of each fraction were calculated (data not shown). In addition, we confirmed the antifungal substances from several types of isolates, such as *Lb. sakei* LBP-S01, *Lc. lactis* LBP-S03, *Ln. mesenteroides* LBP-K06, *W. cibaria* LBP-K15 and *W. confusa* LBP-K16, similar to those from *Lb. plantarum* LBP-K10. Lactic acid bacteria were able to produce and secrete analogous extracellular metabolites (data not shown).

Structural determination of antifungal substances

After we preliminarily confirmed the antifungal activity of seventeen fractions (data not shown), all fractions were identified by GC-MS followed by crystallization to determine the three-dimensional structures of the antifungal compounds. To identify the structures of the antifungal substances from the culture, the fractions with activity against *Ganoderma* mycelium were analyzed by GC-MS using EI

Table 1. Mass analysis of active fractions using EI and CI by GC-MS

Fractions	*m/z* of $[M+H]^+$	*m/z* (%) of EI-MS $[M]^+$	Predicted molecules
F6 (9.5-10.5)*	261.0	261.2 (2.3), 260.2 (12.4), 244.2 (1.2), 224.2 (1.2), 155.1 (8.7), 154.1 (100.0), 153.1 (3.7), 147.1 (1.2), 125.1 (4.1), 114.1 (0.2), 108.1 (4.6), 107.1 (38.7), 91.1 (2.1), 77.1 (3.3), 71.1 (1.4), 70.1 (18.8)	*cis*-cyclo(L-Tyr-L-Pro), $C_{14}H_{16}N_2O_3$
F7 (12.2-13.2)*	197.0	196.2 (9.2), 181.1 (1.9), 169.1 (0.4), 168.1 (0.7), 167.1 (0.8), 156.1 (1.4), 155.1 (16.3), 154.0 (100.0), 153.1 (14.3), 152.1 (2.1), 140.1 (2.3), 139.1 (1.1), 138.1 (6.2), 137.1 (2.1), 125.1 (44.8), 114.1 (3.2), 113.1 (1.1), 112.1 (1.8), 111.1 (1.0), 110.1 (5.3), 107.1 (2.0), 100.1 (0.3), 99.1 (3.2), 98.1 (8.0), 97.1 (3.3), 96.1 (2.4), 84.1 (2.8), 83.1 (2.3), 82.1 (1.8), 76.1 (3.5), 73.1 (5.2), 72.1 (48.0), 71.1 (7.1), 70.1 (86.7), 69.1 (15.2), 68.1 (8.4)	*cis*-cyclo(L-Val-L-Pro), $C_{10}H_{16}N_2O_2$
F9 (15.0-16.5)[a]	185.0	154.0 (3.3), 149.0 (5.2), 141.0 (7.9), 129.0 (9.8), 128.0 (100.0), 127.0 (3.3), 113.0 (20.3), 112.0 (4.2), 99.0 (8.0), 98.0 (4.7), 92.0 (3.7), 91.0 (6.2), 86.0 (15.0), 85.0 (6.4), 84.0 (5.7), 83.0 (3.4), 71.1 (7.0), 70.1 (10.4), 69.0 (7.8), 60.0 (3.8), 57.0 (13.2), 56.0 (6.0), 55.0 (7.4)	*cis*-cyclo(L-Ser-L-Pro), $C_8H_{12}N_2O_3$
F12 (20.5-22.0)*	211.0	210.0 (0.5), 195.0 (0.6), 181.0(1.2), 168.0 (0.6), 167.0 (2.0), 156.0 (0.8), 155.0 (8.8), 154 (100.0), 153.0 (5.3), 125.0 (11.9), 98.0 (2.8), 86.0 (18.2), 70.0 (48.5), 55.0 (3.3)	*cis*-cyclo(L-Leu-L-Pro), $C_{11}H_{18}N_2O_2$

[a] Retention time (min)

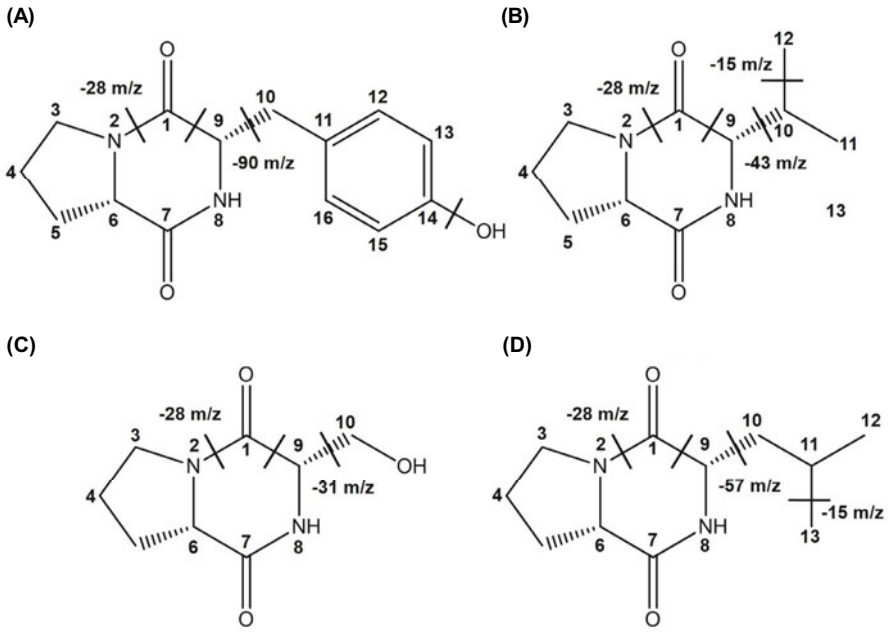

Fig. 2. Proposed structures of active fractions by EI and CI using GC-MS. (A) *m/z* 261 $[M+H]^+$ base peak for F6, (B) *m/z* 197 $[M+H]^+$ base peak for F7, (C) *m/z* 185 $[M+H]^+$ base peak for F9 and (D) *m/z* 211 $[M+H]^+$ base peak for F12. The predicted structures of F6, F7, F9 and F12 were as follows. F6, *cis*-cyclo(L-Tyr-L-Pro); F7, *cis*-cyclo(L-Val-L-Pro); F9, *cis*-cyclo(L-Ser-L-Pro); F12, *cis*-cyclo(L-Leu-L-Pro). The EI and CI values of F17 were reported previously (Kwak *et al.*, 2013). Structural units separated by chemical bonds are divided by dashed lines.

and CI (Table 1). The following active compounds from *Lb. plantarum* LBP-K10 were identified. The *m/z* of the $[M+H]^+$ values of F6, F7, F9, F12, and F17 were identified to be 261, 197, 185, 211, and 245, respectively (Table 1). The EI fragmentation patterns were used to predict the molecular structures of these fractions (Table 1). The EI and CI values of antifungal F17 were reported previously (Kwak *et al.*, 2013). The high-resolution mass determination of F6, F7, F9, F12, and F17 using EI indicated that these fractions were proline-containing diketopiperazines, which had features in common with EI and CI mass spectra of five active fractions with *m/z* 154 (Table 1). Proposed structures of fractions with activity against *Ganoderma* mycelium were suggested (Fig. 2). From this result, F6, F7, F9, and F12 were predicted to be *cis*-cyclo(L-Tyr-L-Pro), $C_{14}H_{16}N_2O_3$, *cis*-cyclo(L-Val-L-Pro), $C_{10}H_{16}N_2O_2$, *cis*-cyclo(L-Ser-L-Pro), $C_8H_{12}N_2O_3$, and *cis*-cyclo(L-Leu-L-Pro), $C_{11}H_{18}N_2O_2$, respectively.

To determine the exact molecular structures of the most active fractions, F7 and F17, we performed elemental analyses of F17, which had antifungal activity against both *G. boninense* and *C. albicans*; the molecular formula that was calculated using the elemental analyses was confirmed to be $C_{14}H_{16}N_2O_2$ (Table 2). We previously confirmed the crystal structure of F17 (Kwak *et al.*, 2013).

Table 2. Elemental analysis of F17 from *Lb. plantarum* LBP-K10

Element	Ratio (%)	Detected mass	Calculated numbers	Estimated numbers	Calculated mass
Carbon	66.9970	163.4727	13.6227	14	168
Nitrogen	11.2623	27.4800	1.9628	2	28
Hydrogen	6.3883	15.5875	15.5874	16	16
Oxygen	13.1100	31.9884	1.9992	2	32
Sulfur	0	0	0	0	0.0000
Total	97.7576	238.5286			244

Finally, to determine the three-dimensional structure of F7, the crystal data, data collection, refinement and phasing statistics of purified F7 were verified to correspond to the molecular formula of $C_{10}H_{16}O_2N_2$, *cis*-cyclo(L-Val-L-Pro) (Fig. 3 and Table 3). In conclusion, all substances with activity against fungal pathogens, including *G. boninense* and

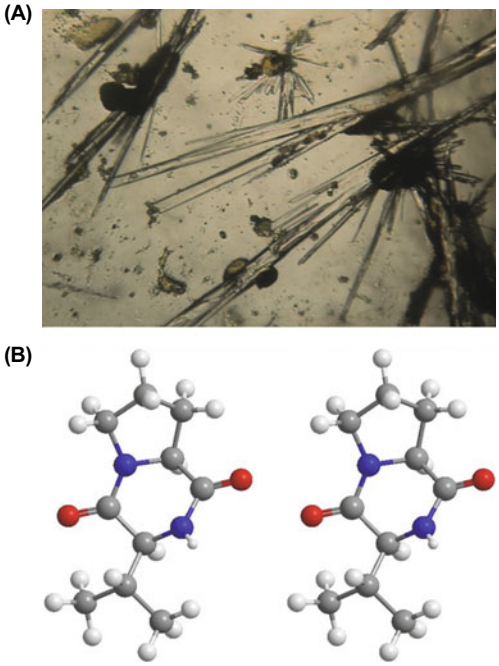

Fig. 3. Three-dimensional structure of F7 determined by X-ray crystallographic analysis. (A) Crystals and (B) stereo presentations of *cis*-cyclo(L-Val-L-Pro). Carbon (grey), hydrogen (white), oxygen (red), and nitrogen (blue) are shown. The three-dimensional structure of F17 was proposed previously (Kwak *et al.*, 2013).

Table 3. Crystal data, data collection, refinement and phasing statistics of F7

cis-cyclo(L-Val-L-Pro)	
Crystal data	
Chemical formula	$C_{20}H_{32}N_4O_4$
M_r	392.50
Crystal system, space group	Orthorhombic, $P2_12_12_1$
Temperature (K)	100(2)
a, *b*, *c* (Å)	5.6240(11), 10.257(2), 34.240(7)
a, *b*, *g* (°)	90, 90, 90
V (Å^3)	1975.1
Z	4
m (mm^{-1})	0.045
F (000)	848
Crystal size (mm)	0.15 × 0.05 × 0.05
Data collection	
Diffractometer	ADSC Quantum 210 2D SMC, PAL-6B
θ_{max} (°)	2.88 to 28.00
No. of measured, independent and observed [I>2s(I)] reflections	21052, 5927
R_{int}	0.0604
Refinement	
$R[F^2>2s(F^2)]$, $wR(F^2)$, S(all data)	0.0324, 0.0822, 0.0338, 0.0836
No. of reflections	5927
No. of parameters	284
No. of restrains	0
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement
Dr_{max}, Dr_{min} (e Å^{-3})	0.369, -0.279

C. albicans, were determined to be 1,4-diaza-2,5 dioxo-cyclic forming dipeptides, i.e., the so-called modified 2,5-dioxopiperazines, 2,5-diketopiperazines or *cis*-cyclodipeptides.

Antifungal activity of cyclic dipeptides

Our experiments indicated that the anti-*Ganoderma* activities of *cis*-cyclo(L-Tyr-L-Pro), *cis*-cyclo(L-Val-L-Pro), *cis*-cyclo (L-Ser-L-Pro), *cis*-cyclo(L-Leu-L-Pro), and *cis*-cyclo(L-Phe-L-Pro) were significantly higher than those of the other fractionated compounds, and their corresponding concentrations were 8.2, 8.1, 9.0, 8.4, 6.8 mM, respectively (Fig. 4A). In particular, *cis*-cyclo(L-Val-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) showed remarkable activity against *G. boninense* compared with that of the other fractionated compounds (Fig. 4A). We also tested the fractions for antifungal activities against the opportunistic human pathogenic fungi. In contrast to the results for *G. boninense*, only *cis*-cyclo(L-Phe-L-Pro) demonstrated prominent anti-*Candida* activity at a concentration of 6.8 mM; this concentration was similar to that required for activity against *Ganoderma* mycelium (Fig. 4B).

Discussion

Using preliminary experiments, we determined that fractions F6, F7, F9, F12, and F17 were representative of the anti-*Ganoderma* fractions, and these fractions increased significantly between 56 h and 72 h (data not shown). It was inferred that antifungal compounds might be produced as secondary metabolites during bacterial growth despite the decrease in cell numbers to control the extracellular environment and their own metabolism.

The isolated *Lb. plantarum* LBP-K10, which was the most potent antifungal strain among the isolates from Korean kimchi, was identical to *Lb. plantarum* WCFS1, which might be able to dissipate pyruvate. The latter produces various fermentation end-products associated with their metabolism, which is driven by the relatively abundant regulatory phosphoenolpyruvate-dependent sugar phosphotransferase system and stress-related proteins, in contrast to other lactic acid bacteria (Mayo *et al.*, 2008). During the normal growth of lactic acid bacteria, cells are believed to excrete many secondary metabolites for their own use. Therefore, culture filtrate was chosen for investigating the potential of small molecules to act as antifungal compounds. This decision was based on previous studies of the stability against heat, pH and proteolytic enzymes with antagonistic activity (Niku-Paavola *et al.*, 1999; Ström *et al.*, 2002; Nardi *et al.*, 2005).

Using methylene chloride extraction, we selectively isolated several cyclic dipeptides from the culture filtrate as reported previously (Kwak *et al.*, 2013). Of the isolated cyclic dipeptides, the most potent anti-*Ganoderma* compounds were *cis*-cyclo(L-Val-L-Pro) and *cis*-cyclo(L-Phe-L-Pro). These compounds were identified by GC-MS using EI and CI (Fig. 2 and Table 1), Elemental analysis (Table 2) and the identities

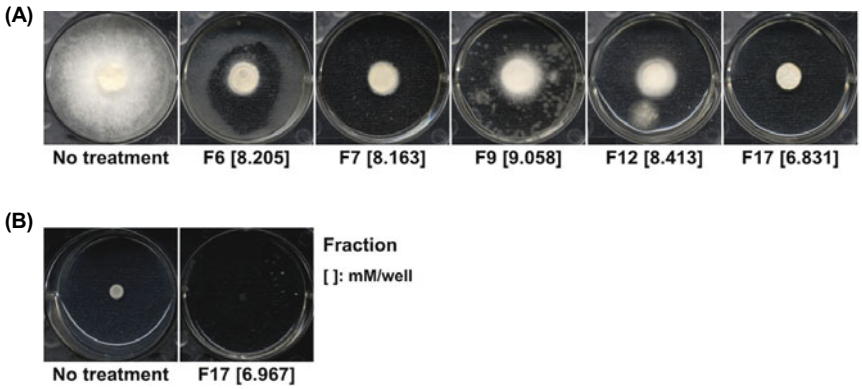

Fig. 4. Antifungal activity of cyclic dipeptides. (A) The antifungal activity of five fractions from *Lb. plantarum* LBP-K10 was suggested. The initial inoculation of *Ganoderma* cells was obtained from a 7-day culture of *Ganoderma* mycelium. (B) The antifungal activity of *cis*-cyclo(L-Phe-L-Pro) from *Lb. plantarum* LBP-K10 was observed. All experimental procedures were as described in the 'Materials and Methods'.

were confirmed using X-ray crystallography (Table 3). The *Lb. plantarum* genome has been shown to encode the pathways for synthesizing most amino acids except for branched-chain amino acids, such as valine, leucine and isoleucine (Mayo *et al*., 2008). However, the following factors must be considered for most cyclic dipeptides, including the branched-amino acids from the culture supernatant of *Lb. plantarum* LBP-K10. One is that cyclic dipeptides are supposedly produced from exogenous amino acids, dipeptides or polypeptides in the culture media. The other is that a gene cluster related to the synthesis of a non-ribosomal peptide was revealed for the first time in lactic acid bacterial strains using the genome data base (Mayo *et al*., 2008).

In this study, the active concentrations of *cis*-cyclo(L-Tyr-L-Pro), *cis*-cyclo(L-Val-L-Pro), *cis*-cyclo(L-Ser-L-Pro), *cis*-cyclo(L-Leu-L-Pro), and *cis*-cyclo(L-Phe-L-Pro) against *G. boninense* were 2.13, 1.6, 1.67, 1.77, and 1.67 mg/ml, respectively. In addition, 1.7 mg/ml *cis*-cyclo(L-Phe-L-Pro) was active against *C. albicans*. These active concentrations were lower than the 20 mg/ml cyclo(Phe-Pro) concentration that was measured against *Aspergillus fumigatus* and *Penicillium roqueforti* in a previous study (Ström *et al*., 2002). Furthermore, the antifungal activity of *cis*-cyclo(L-Phe-L-Pro), which was termed M17 and isolated from *Ln. mesenteroides* LBP-K06 in this study, was almost the same as that of the *cis*-cyclo (L-Phe-L-Pro) isolated from *Lb. plantarum* LBP-K10 (data not shown). Investigations of the HPLC profiles from various lactic acid bacteria suggested that lactic acid bacteria produce and excrete analogous metabolites that function as antifungal substances in their own culture filtrate (data not shown).

We focused not only on single molecules as fungicidal effectors but also on a synergistic complex of cyclic dipeptides (data not shown). The culture filtrate without organic acids which included cyclic dipeptides, such as *cis*-cyclo(L-Tyr-L-Pro), *cis*-cyclo(L-Val-L-Pro), *cis*-cyclo(L-Ser-L-Pro), *cis*-cyclo(L-Leu-L-Pro), and *cis*-cyclo(L-Phe-L-Pro), was suggested to contain anti-*Ganoderma* compounds (data not shown). Because combinations of the antifungal compounds revealed synergistic effects (Rhee, 2004; Lee *et al*., 2010), the synergistic activity of cyclic dipeptides can be developed and utilized to eliminate fungal pathogens. Therefore, the minute control of the culture filtrate of lactic acid bacteria, which is rich in antimicrobial materials, should be considered. Moreover, the compounds *cis*-cyclo(L-Val-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) from the culture filtrate of *Lb. plantarum* LBP-K10 that demonstrated antifungal activity against *G. boninense* and *C. albicans* have been submitted in patents application (Republic of Korea Patent Application no. 10-2011-0064091, 2011; PCT/KR2011/004770, 2011; Republic of Korea Patent Application no. 10-2013-0105346, 2013).

Acknowledgements

The authors thank Cilic BioEngineering Sdn Bhd (CBE) in Taman Tawau, Sabah in Malaysia for supplying *G. boninense* isolate (GMR3). We also thank NCIRF at Seoul National University for supporting GC-MS experiments.

References

Abee, T., Krockel, L., and Hill, C. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int. J. Food. Microbiol.* **28**, 169–185.

Bivi, M.R., Farhana, M.S.N., Khairulmazmi, A., and Idris, A. 2010. Control of *Ganoderma boninense*: A causal agent of basal stem rot disease in oil palm with endophyte bacteria *in vitro*. *Int. J. Agric. Biol.* **12**, 833–839.

Budesinsky, M., Cisarova, I., Podlaha, J., Borremans, F., Martins, J.C., Waroquier, M., and Pauwels, E. 2010. Structures of cyclic dipeptides: an X-ray and computational study of *cis*- and *trans*-cyclo(Pip-Phe), cyclo(Pro-Phe) and their N-methyl derivatives. *Acta Crystallogr. B.* **66**, 662–677.

Chong, K.P., Rossall, S., and Atong, M. 2009. *In vitro* antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma Boninense*. *J. Agr. Sci.* **1**, 15–20.

Gänzle, M.G., Höltzel, A., Walter, J., Jung, G., and Hammes, W.P. 2000. Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. *Appl. Environ. Microbiol.* **66**, 4325–4333.

Gibson, G.R., Saavedra, J.M., MsFarlane, S., and McFarlane, G.T. 1997. Probiotics and intestinal infections. *In* Fuller, R. (ed.) Probiotics 2: Applications and practical aspects, pp. 10–39. Chapman & Hall, New York, N.Y., USA.

Graz, M., Hunt, A., Jamie, H., Grant, G., and Milne, P. 1999. Antimicrobial activity of selected cyclic dipeptides. *Pharmazie* **54**, 772–775.

Havenaar, R., Brink, B., and Huis in't Veld, J.H.J. 1992. Selection of strains for probiotic use. *In* Fuller. R. (ed.). Probiotics, the scientific basis, pp. 209–224. Chapman and Hall, London, USA.

Holzapfel, W.H., Haberer, P., Geisen, R., Björkroth, J., and Schillinger, U. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am. J. Clin. Nutr.* **73**, 365S–373S.

Kwak, M.K., Liu, R., Kwon, J.O., Kim, M.K., Kim, A.H., and Kang, S.O. 2013. Cyclic dipeptides from lactic acid bacteria inhibit proliferation of the influenza A virus. *J. Microbiol.* **51**, 836–843.

Lee, K.H., Kim, K.W., and Rhee, K.H. 2010. Identification of *Streptomyces* sp. KH29, which produces an antibiotic substance processing an inhibitory activity against multidrug-resistant *Acinetobacter baumannii*. *J. Microbiol. Biotechnol.* **20**, 1672–1676.

Li, H., Liu, L., Zhang, S., Cui, W., and Lv, J. 2012. Identification of antifungal compounds produced by *Lactobacillus casei* AST18. *Curr. Microbiol.* **65**, 156–161.

Mayo, B., van Sinderen, D., and Ventura, M. 2008. Genome analysis of food grade lactic acid-producing bacteria: from basics to applications. *Curr. Genomics* **9**, 169–183.

McCleland, K., Milne, P.J., Lucieto, F.R., Frost, C., Brauns, S.C., Van De Venter, M., Du Plessis, J., and Dyason, K. 2004. An investigation into the biological activity of the selected histidine-containing diketopiperazines cyclo(His-Phe) and cyclo(His-Tyr). *J. Pharm. Pharmacol.* **56**, 1143–1153.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., and Schnürer, J. 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiol. Lett.* **219**, 129–135.

Nardi, R.M., Santoro, M.M., Oliveira, J.S., Pimenta, A.M., Ferraz, V.P., Benchetrit, L.C., and Nicoli, J.R. 2005. Purification and molecular characterization of antibacterial compounds produced by *Lactobacillus murinus* strain L1. *J. Appl. Microbiol.* **99**, 649–656.

Niku-Paavola, M.L., Laitila, A., Mattila-Sandholm, T., and Haikara, A. 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* **86**, 29–35.

O'Neill, J.C. and Blackwell, H.E. 2007. Solid-phase and microwave-

assisted syntheses of 2,5-diketopiperazines: small molecules with great potential. *Comb. Chem. High Throughput Screen.* **10**, 857–876.

Pilotti, C.A., Sanderson, F.R., Aitken, E.A., and Armstrong, W. 2004. Morphological variation and host range of two *Ganoderma* species from Papua New Guinea. *Mycopathologia* **158**, 251–265.

Pilotti, C.A. 2005. Stem rots of oil palm caused by *Ganoderma boninense*: pathogen biology and epidemiology. *Mycopathologia* **159**, 129–137.

Prasad, C. 1995. Bioactive cyclic dipeptides. *Peptides* **16**, 151–164.

Rees, R.W., Flood, J., Hasan, Y., and Cooper, R.M. 2007. Effects of inoculum potential, shading and soil temperature on root infection of oil palm seedlings by the basal stem rot pathogen *Ganoderma boninense*. *Plant Pathol*. **56**, 862–870.

Rhee, K.H. 2004. Cyclic dipeptides exhibit synergistic, broad spectrum antimicrobial effects and have anti-mutagenic properties. *Int. J. Antimicrob. Agents* **24**, 423–427.

Sherman, F. 2002. Getting started with yeast. *Methods Enzymol.* **350**, 3–41.

Ström, K., Sjögren, J., Broberg, A., and Schnürer, J. 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.* **68**, 4322–4327.

Saavedra, J.M. 1995. Microbes to fight microbes: a not so novel approach to controlling diarrheal disease. *J. Pediatr. Gastroenterol. Nutr.* **21**, 125–129.

Trabocchi, A., Scarpi, D., and Guarna, A. 2008. Structural diversity of bicyclic amino acids. *Amino Acids* **34**, 1–24.

Wang, H., Yan, Y., Wang, J., Zhang, H., and Qi, W. 2012. Production and characterization of antifungal compounds produced by *Lactobacillus plantarum* IMAU10014. *PLoS One* **7**, e29452.