Journal of Microbiology (2013) Vol. 51, No. 6, pp. 836–843
DOI 10.1007/s12275-013-3521-y

Cyclic Dipeptides from Lactic Acid Bacteria Inhibit Proliferation of the Influenza A Virus

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(Received October 8, 2013 / Accepted November 30, 2013)

We isolated *Lactobacillus plantarum* LBP-K10 from the traditional Korean fermented food kimchi. When organic acids were removed, the culture filtrate of this isolate showed high antiviral activity (measured using a plaque-forming assay) against the influenza A (H3N2) virus. Two fractions that were active against influenza A virus were purified from the culture filtrate using a C18 column with high-performance liquid chromatography. These active fractions were crystallized and identified to be the cyclic dipeptides *cis*-cyclo (L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) using gas chromatography-mass spectrometry; this identification was confirmed by X-ray crystallography. These cyclic dipeptides were identified in the culture filtrate of other lactic acid bacteria, including *Lactobacillus* spp., *Leuconostoc* spp., *Weissella* spp., and *Lactococcus lactis*.

Keywords: *Lactobacillus plantarum* LBP-K10, culture filtrate, *cis*-cyclo(L-Leu-L-Pro), *cis*-cyclo(L-Phe-L-Pro), influenza A virus, antiviral activity

Introduction

Lactic acid bacteria and their culture supernatants are useful tools for inhibiting bacteria and fungi (Rouse and van Sinderen, 2008). The repression of yeast growth in the rodent stomach (Savage, 1969) and the antagonistic effect on the *Ristella* species in the murine digestive tract by lactic acid bacteria have been reported (Ducluzeau *et al*., 1971). A fermented mixture containing *Lactobacillus acidophilus* inhibited the growth of *Shigella dysenteriae*, *Salmonella typhimurium*, and *Escherichia coli* (Rani and Khetarpaul, 1998). *Cryptosporidium parvum* infection was suppressed in C57BL/6 female mice by an inoculation with *Lactobacillus reuteri* (Alak *et al*., 1997). The antifungal activities of *Lb. acidophilus, Lb. reuteri*, *Lactobacillus casei* GG, and *Bifidobacterium animalis* were investigated by measuring their capacities to protect athymic and euthymic mice from systemic candidiasis (Wagner *et al*., 1997). *Lactobacillus coryniformis*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus* were also active against fungi (Magnusson *et al*., 2003). *Lactococcus lactis*, *Streptococcus cremoris* R3, *Lactococcus diacetylactis* V1, and *Streptococcus thermophilus* T2 strains showed a wide inhibitory spectrum against Gram-positive bacteria (Mezaini *et al*., 2009). Lactic acid bacteria isolated from rye sourdoughs were reported to produce bacteriocin-like inhibitory substances against bacteria and fungi (Digaitiene *et al*., 2012).

The antimicrobial activities of lactic acid bacteria are thought to be resulted from the production of low-molecular weight substances, including lactic acid, acetic acid, hydrogen peroxide, amino acids, diacetyl, 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 (Niku-Paavola *et al*., 1999; Gänzle *et al*., 2000; Ross *et al*., 2002; Ström *et al*., 2002; Li *et al*., 2012). Cyclo(Gly-Leu) from *Lb. plantarum* VTT E-78076 inhibited the growth of the *Pantoea agglomerans* VTT E-90396 and plant pathogenic fungi, *Fusarium avenaceum* VTT-D-80147 (Niku-Paavola *et al*., 1999). Cyclo(Leu-Pro) from *Lb. casei* AST18 (Li *et al*., 2012) and cyclo(Phe-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) from *Lb. plantarum* MiLAB 393 (Ström *et al*., 2002) were reported to be antifungal substances. The synergistic antibacterial activity of cyclo(Phe-Pro) and cyclo(Leu-Pro) from the *Streptomyces* species has been reported against bacteria, even against the vancomycin-resistant *Enterococcus faecium*, *Candida albicans*, and *Cryptococcus neoformans* (Rhee, 2004). Cyclo(Trp-Trp) from *Streptomyces* sp. KH29 showed inhibitory activity against the multidrug-resistant *Acinetobacter baumannii* and against other microbial strains (Lee *et al*., 2010). Cyclo(Phe-Phe) inhibited the serotonin transporter and acetylcholinesterase *in vitro*, acting to prevent the development of Alzheimer's disease and dementia (Tsuruoka *et al*., 2012). Cyclo(Phe-Pro), and cyclo(Tyr-Pro) from *Lb. reuteri* were identified as signaling effectors in quorum sensing by attenuating the virulence factor produced by staphylococci (Li *et al*., 2011). Glycine-containing cyclic dipeptides, cyclo(Phe-Cys) and especially cyclo(Tyr-Cys), significantly inhibited cancer cell growth in HT-29, HeLa, and MCF-7 cells *in vitro* (Van der Merwe *et al*., 2008). Cyclo(Pro-Trp), cyclo(Phe-Pro), cyclo (Trp-Pro), and cyclo(Trp-Trp) showed the growth inhibition of microbes and enhanced the maturation of gastrointestinal cells (Graz *et al*., 1999). Cyclo(His-Phe) and cyclo(His-Tyr)

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were significantly active against cardiovascular disease and cervical carcinoma cells (McCleland *et al*., 2004). In addition, an albonoursin-producing strain, *Streptomyces albulus* KO-23, was reported to catalyze the conversion of cyclic dipeptides into the corresponding dehydro derivatives, which have been observed to interfere with cell division (Kanzaki *et al*., 2000).

Cyclic dipeptides and their structures have been widely studied in terms of their biological activities, including their activities against bacteria and fungi (Prasad, 1995; Trabocchi *et al*., 2008); however, their antiviral activity has not been reported yet. Therefore, we isolated lactic acid bacteria from the Korean fermented food kimchi to find antiviral substances from their culture supernatants.

Materials and Methods

Strain

Lactic acid bacteria were isolated from naturally fermented plants including various vegetables, such as Chinese cabbage, mustard leaves and stems, and stonecrop, on de Man, Rogosa and Sharpe (MRS) medium (De Man *et al*., 1960) including 1.0% agar. To identify the isolates, the 16S rDNA sequencing method was used. Eubacterial primers were used for polymerase chain reaction (PCR) (27F: 5′-AGA GTT TGA TCM TGG CTC AG-3′, 1492R: 5′-GGY TAC CTT GTT ACG ACT T-3′). The following reactions were used for PCR: 30 cycles, pre-denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 7 min. The homologies of the selected 16S rDNA sequences resulting from the isolated strains were compared using a nucleotide BLAST (NCBI, http://www.ncbi.nlm.nih.gov) to identify the amplified PCR products using the genome database. All isolated lactic acid bacteria were cultivated using MRS medium at 30°C for 3 days. To predict antiviral activity, the antibacterial and antifungal activities were measured using the minimum inhibitory concentration and disk diffusion assay.

Preparation of culture filtrate without organic acids

To avoid the interference between antiviral substances and organic acids from the culture filtrate of *Lb. plantarum* LBP-K10, the organic acids was removed through the weak anion exchanger Amberlite IRA-67 as previously proposed with some modifications (Gao *et al*., 2010). For HPLC analysis, L-lactic acid, acetic acid, butyric acid and propionic acid were purchased (Sigma, USA) to quantify the organic acids that were removed by elution through Amberlite IRA-67 resins. The eluents from the culture supernatant were lyophilized, and the resulting powders were extracted with 5-fold volumes of methylene chloride. After the methylene chloride fraction was evaporated, the resulting powder was dissolved in the appropriate amount of sterilized distilled water for plaque assays.

Fractionation of antiviral substances

The supernatant from the three-day cultured *Lb. plantarum* LBP K-10 was concentrated by lyophilization and extracted with methylene chloride. After the elimination of methylene chloride by evaporation, the syrupy resultant was dissolved in distilled water and filtered with a 0.22 μm-cellulose acetate membrane. The filtered samples were separated by a high-performance liquid chromatography (semi-prep HPLC system, Agilent 1200 series, USA) system with a semi-preparative C18 reverse-phase column (9.4 × 250 mm, Agilent, USA) and the ChemStation HPLC software. The mobile phase was 67.0% water, 3.0% acetonitrile and 30.0% methanol for 45 min, and the wavelengths for observing the corresponding chromatograms were 210, 260, and 280 nm, respectively. Each fraction was collected and concentrated by lyophilization to obtain a powder.

Plaque assay

Antiviral activity was investigated by a previously proposed procedure with some modifications (Huprikar and Rabinowitz, 1980). Madin-Darby canine kidney (MDCK) epithelial cells were used as host cells, and the use of the influenza A (H3N2) virus for infection was supported by Korea National Institute of Health. Crystallized single compounds used for X-ray crystallography were used for the plaque assay in this study. In addition, to compare the antiviral substances to other analogues, we purchased cyclo(Met-Pro) and cyclo (Val-Pro) (Bachem, Switzerland) for the plaque assay.

Cytotoxicity test

The cytotoxic effects of the cyclic dipeptides on MDCK cells were determined using Mosmann-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide according to a previously proposed method (Watanabe *et al*., 1994). MDCK cells were inoculated on 96-well round-bottomed microtiter plates at an appropriate density of cells. Cells grown in Dulbecco's modified Eagles's medium (DMEM) with 10% fetal bovine serum were incubated at 37°C. After the MDCK cells were grown in DMEM for 48 h, each well was treated with cyclic dipeptides in concentrations ranging from 2.0 to 14.0 mM (2.0 mM unit), and the cells were incubated for another 48 h. The cell viability was assessed by a microtiter plate reader using a Power Wave X spectrophotometer (Bio-tek Instruments, USA) by measuring absorbance at 492 nm.

Mass spectrometry

To investigate the electron ionization (EI) and chemical ionization (CI) of each fraction, the molecular mass was measured by gas chromatography-mass spectrometry (GC-MS) system using a direct insertion probe. A chromatographic system consisting of an Agilent 6890 series GC (Agilent Technologies, Germany) equipped with a 7679 series automatic liquid sampler was used. Mass spectrometric analyses were conducted using a high-resolution mass spectrometer (JEOL JMS-700, Japan).

X-ray crystallography

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-

Table 1. Adsorption of organic acids using a weak anion exchanger, Amberlite IRA-67

Substance	Quantity (g/L)	Adsorbed rate (%)	Not adsorbed rate (%)
Organic acid			
lactic acid	6.78 (± 0.57)	96.88	3.12
acetic acid	1.19 (± 0.11)	99.6	0.4
butyric acid	0.73 (± 0.08)	100	0
propionic acid	0.65 (± 0.14)	100	0

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.cdc.am.c.uk/ervices/structure_deposit/). The data have been assigned to deposition numbers CCDC 937533 and CCDC 937534, which correspond to the 13th and 17th fractions, respectively.

Results

Isolation and identification of lactic acid bacteria from kimchi

Approximately 400 strains of lactic acid bacteria, including *Leuconostoc* spp., *Lactobacillus* spp., *Weissella* spp., and *L. lactis*, were isolated from kimchi that was prepared using various vegetables as described. These strains were identified using 16S rDNA sequencing methods with PCR amplification (data not shown). Among the isolates, *Lb. plantarum* LBP-K10 had a culture filtrate that preliminarily showed the most significant antibacterial and antifungal activity (data not shown). The culture filtrate from the three-day cultured *Lb. plantarum* LBP-K10 showed a significant antibacterial and antifungal activity, which was confirmed for several conditions; the culture filtrate was stable against heat and proteolysis (data not shown). Taken together, these data suggested that *Lb. plantarum* LBP-K10 should be used in this study to isolate the antiviral substances against influenza A virus.

Table 2. Antiviral activity of the culture filtrate without organic acids

Sample	Sample ratio (%) / Quantity (mg/ml)	Plaque number (±SD)	Inhibition ratio (%)
Untreated cells	0 / 0	76.6 (± 5.7)	0
Cells treated with the culture filtrate without organic acids	0.5 / 7.176	71 (± 2.6)	7.3
	1.0 / 14.352	46.3 (± 4.6)	39.6
	1.5 / 21.528	34.6 (± 6.1)	54.9
	2.0 / 28.704	25 (± 2.6)	67.4

Antiviral activity of the culture filtrate without organic acids

Because of the large amount of organic acids in the culture filtrate of the isolates, the antiviral activity had to be con-

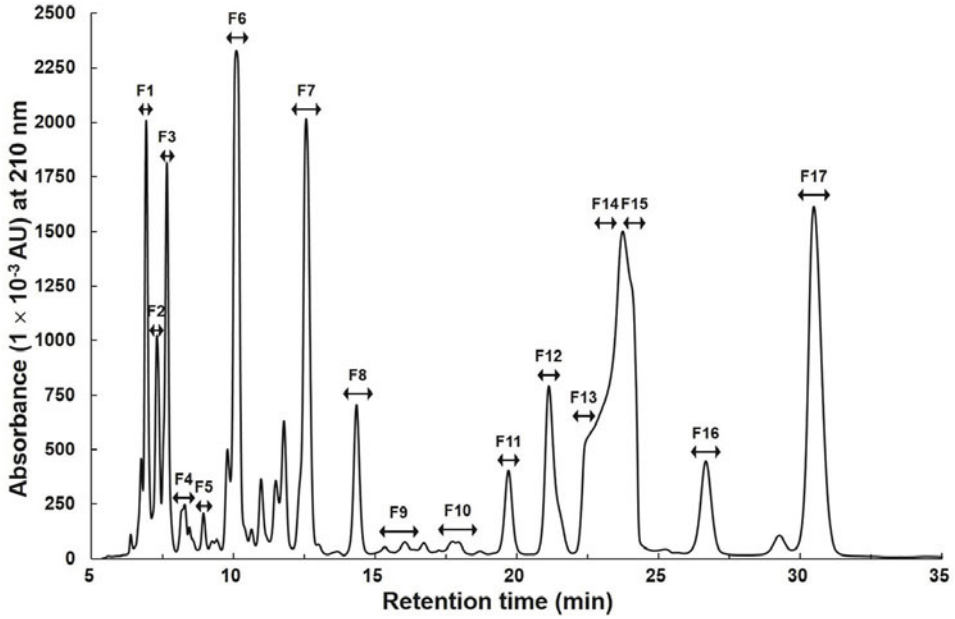

Fig. 1. Seventeen fractions from the culture filtrate were collected by methylene chloride extraction. Seventeen different fractions were collected from the HPLC profiles; these fractions were separated according to their retention times. AU, arbitrary unit.

Table 3. Mass analysis of fractions by EI and CI using GC-MS

Fractions	m/z of $[M+1]^+$	m/z (%) of EI-MS	Predicted molecules
F13 (22.5-23.5)*	211.0	166.1 (10.7), 154.1 (33.9), 148.0 (30.4), 135.1 (11.4), 125.1(3.0), 121.1 (12.2), 105.0 (19.0), 103.1 (13.2), 91.0 (100.0), 87.1 (66.0), 77.1 (13.5), 76.0 (40.2), 75.1 (22.5), 71.1 (7.7), 70.1 (20.9), 69.1 (76.2), 65.1 (12.1), 57.1 (20.7), 55.0 (7.0), 51.0 (6.4)	*cis*-cyclo(L-Leu-L-Pro)
F17 (30.0-31.0)[a]	245.0	244.1 (97.5), 153.0 (82.0), 125.1 (100.0), 91.0 (52.3), 70.1 (46.3)	*cis*-cyclo(L-Phe-L-Pro)

[a] Retention time (min)

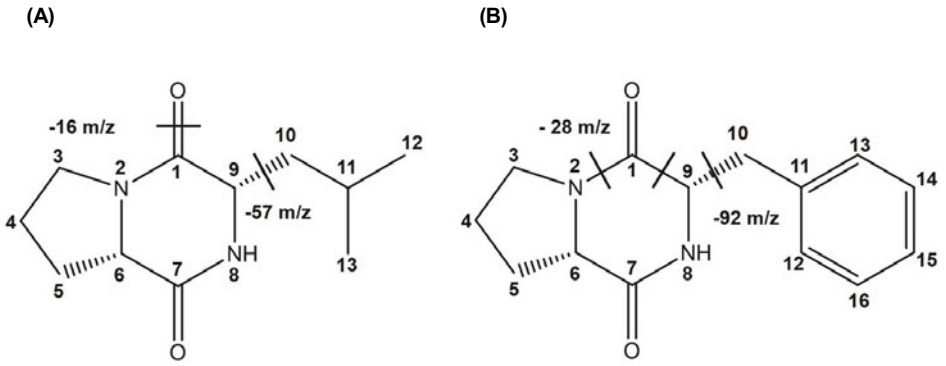

Fig. 2. Proposed structures of F13 and F17 using GC-MS. EI and CI mass spectra revealed (A) *m/z* 211 $[M+H]^+$ base peak for F13 and (B) *m/z* 245 $[M+H]^+$ base peak for F17. The predicted structures of F13 and F17 were as follows. F13: *cis*-cyclo(L-Leu-L-Pro) and F17: *cis*-cyclo(L-Phe-L-Pro). Structural units separated by chemical bonds are divided by dashed lines.

sidered and predicted after removing organic acids. Antiviral activity using the culture filtrate was tested without organic acids for the following reasons: the broad spectra of antimicrobial and antiviral activity of lactic acid and other volatile acids originating from lactic acid bacteria have previously been reported (Naidu *et al*., 1999). Also, the MDCK cells for the plaque assay cannot grow in the presence of organic acids (data not shown). The organic acids were completely removed by using Amberlite IRA-67 resins, and their quantities are shown in Table 1. After the elimination of organic acids from the culture filtrate, the antiviral activity against the influenza A (H3N2) virus was investigated; the viral proliferation with the culture filtrate was inhibited approximately 70% of the viral proliferation without the culture filtrate (Table 2). From this result, it was inferred that the antiviral compounds of the culture filtrate without organic acids could contribute to the inhibition of both plaque-forming viral infections and the proliferation of the influenza A virus.

Fractionation of the culture filtrate

The highest antiviral activity was observed in the culture filtrate of the three-day cultured cells, and this activity was maintained for 2-3 years under cold-storage conditions (data not shown); therefore, the anti-influenza A viral compounds were purified from the culture filtrate of the three-day cultured cells. The antiviral activity of the culture filtrate was maintained even after sterilization and filtration through a 0.22 μm-cellulose acetate membrane, suggesting that the antiviral substances are small compounds. Seventeen different fractions, designated F1-F17, were collected from the culture filtrate of *Lb. plantarum* LBP-K10 (Fig. 1).

Identification, antiviral activity and cytotoxicity of active fractions

After we preliminarily confirmed the antiviral activity of seventeen fractions (data not shown), all fractions were identified by GC-MS followed by crystallization to deter-

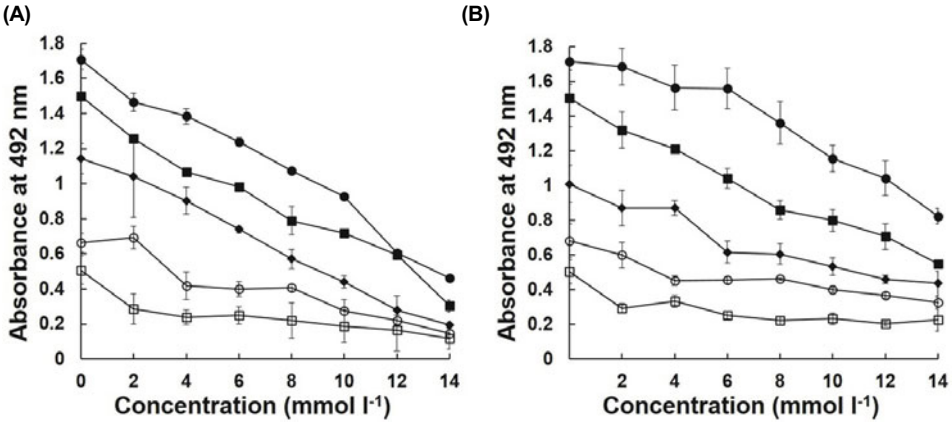

Fig. 3. Cytotoxic effect of antiviral compounds on MDCK cell. Cytotoxicity was measured with *cis*-cyclo(L-Leu-L-Pro) (A) or *cis*-cyclo(L-Phe-L-Pro) (B) using MDCK cells. The endpoint viability assay was utilized for 96-well microplates for high-throughput screening method using the MTT assay. Each experiment was investigated with concentrations ranging from 2.0 mM to 14.0 mM and was incubated for 48 h. The following initial cell numbers were used: 6.0×10^4 cells (●), 3.0×10^4 cells (■), 1.5×10^4 cells (◆), 7.5×10^3 cells (○), and 3.75×10^3 cells (□). All results are expressed as the means ± standard deviation of triplicate experiments.

Table 4. Anti-influenza A (H3N2) viral activity by plaque assays

	Concentration (mM)	Plaque number[a] (±SD)	Inhibition ratio (%)
Untreated cells	0	154.3 (±7.6)	0
Cyclo(Met-Pro)-treated cells	5.0	151.0 (±8.0)	2.1
	10.0	128.7 (±7.7)	16.6
Cyclo(Val-Pro)-treated cells	5.0	145.0 (±3.2)	6.0
	10.0	141.7 (±4.9)	8.2
F13-treated cells	2.5	147.3 (±5.5)	4.5
	5.0	0	100
F17-treated cells	2.5	108.3 (±8.5)	29.8
	5.0	0	100

[a] Plaque numbers were expressed as the Means±SD of at least three independent experiments.

mine the three-dimensional structures of the antiviral compounds. Therefore, we examined the antiviral activity with crystallized samples after they were dissolved in an appropriate amount of sterilized distilled water. Of the 17 fractions, CI values of the antiviral fractions F13 and F17 were revealed to be 211 and 245, respectively; the EI fragmentation pattern suggested that F13 and F17 were *cis*-cyclo (L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro), respectively (Fig. 2 and Table 3). The concentrations of the antiviral activities of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) were observed to be greater than 2.5 mM, and no plaques were observed even after 60 h in 5.0 mM-treated MDCK cells infected with influenza virus (Table 4). By contrast, no antiviral activity was detected for commercially purchased proline-containing cyclic dipeptides, such as cyclo(Met-Pro) and cyclo(Val-Pro) (Table 4). Furthermore, the concentrations of cyclo(Met-Pro) and cyclo(Val-Pro) were approximately twice the concentrations of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro). This result suggests that analogues of cyclic dipeptides could be used as infectivity references to account for the host cell viability and the infectivity. However, the antiviral activities of two cyclic dipeptides, *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro), demonstrated some cytotoxicity depending on their concentrations (Fig. 3). High concentrations, especially concentrations above 10.0 mM, of the two cyclic dipeptides slightly affected the viability of the host cells (Fig. 3). Because their effective concentrations against influenza virus were much lower than the cytotoxic concentrations against host cells, we concluded that concentrations from 2.5-5.0 mM significantly inhibited the viral infectivity and proliferation (Fig. 3 and Table 4).

Structural determination of antiviral substances

To confirm the structures of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro), X-ray crystallography was performed using these antiviral compounds (Table 5, 6, and Fig. 4). Crystals of *cis*-cyclo(L-Leu-L-Pro) (Fig. 4A, left) or *cis*-cyclo (L-Phe-L-Pro) (Fig. 4B, left) were obtained using methanol/methylene chloride. The three-dimensional structures of *cis*-cyclo(L-Leu-L-Pro) (Fig. 4A, right) and *cis*-cyclo(L-Phe-L-Pro) and (Fig. 4B, right) were proposed from the X-ray diffraction data (Tables 5 and 6). Two antiviral compounds

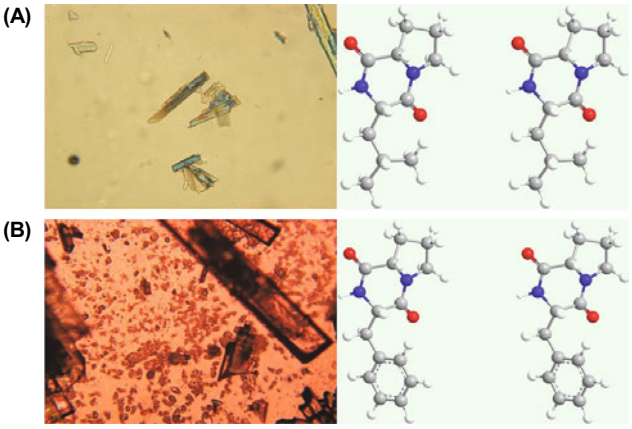

Fig. 4. Three-dimensional structures of *cis*-cyclo(L-Leu-L-Pro) (A) and *cis*-cyclo(L-Phe-L-Pro) (B) determined by X-ray crystallographic analysis. Crystals (A and B, left) and stereo presentations (A and B, right) of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro). Carbon (grey), hydrogen (white), oxygen (red), and nitrogen (blue) are indicated.

from *Lb. plantarum* LBP-K10 were confirmed to be *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) (Tables 5, 6, and Fig. 4).

Table 5. Crystal data, data collection, refinement, and phasing statistics of F13

cis-cyclo(L-Leu-L-Pro)	
Crystal data	
Chemical formula	$C_{11}H_{18}N_2O_2$
M_r	210.277
Crystal system, space group	Orthorhombic, $P2_12_12_1$
Temperature (K)	100
a, b, c (Å)	6.2820, 9.2440, 19.6250
a, b, g (°)	89.998, 90.003, 90.022
V (Å^3)	1139.64
Z	4
m (mm^{-1})	0.08
F (000)	456
Crystal size (mm)	0.1 × 0.3 × 0.5
Data collection	
Diffractometer	ADSC Quantum 210 CCD, PAL-6B
q_{max} (°)	
No. of measured, independent and observed [I>2s(I)] reflections	4345, 1399, 1362
R_{int}	0.0543
Refinement	
$R[F^2>2s(F^2)]$, $wR(F^2)$, S(all data)	0.1120, 0.2878, 2.8194
No. of reflections	1399
No. of parameters	136
No. of restrains	0
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}/\Delta\rho_{min}$ (e Å^{-3})	1.18, -0.40

Table 6. Crystal data, data collection, refinement and phasing statistics of F17

cis-cyclo (L-Phe-L-Pro)	
Crystal data	
Chemical formula	$C_{14}H_{16}N_2O_2$
M_r	244.12
Crystal system, space group	Monoclinic, $P2_1$
Temperature (K)	100
a, b, c (Å)	5.592, 10.008, 10.681
a, b, g (°)	90, 87.634, 90
V (Å^3)	597.25
Z	2
m (mm^{-1})	0.089
F (000)	260
Crystal size (mm)	0.1 × 0.3 × 0.5
Data collection	
Diffractometer	ADSC Quantum 210 CCD, PAL-6B
θ_{max} (°)	27
No. of measured, independent and observed [I>2s(I)] reflections	2220, 1314, 1254
R_{int}	0.069
Refinement	
$R[F^2>2s(F^2)]$, $wR(F^2)$, S(all data)	0.1019, 0.2667, 2.519
No. of reflections	1312
No. of parameters	164
No. of restrains	1
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}/\Delta\rho_{min}$ (e Å^{-3})	1.09, -0.44

Discussion

Although more than one hundred cyclic dipeptides have been reported to have biological activities recently (Prasad, 1995; Trabocchi *et al.*, 2008), researchers have focused mainly on the antibacterial and antifungal characteristics (Niku-Paavola *et al.*, 1999; Gänzle *et al.*, 2000; Ström *et al.*, 2002; Rhee, 2004; Lee *et al.*, 2010; Li *et al.*, 2012). However, the cyclic dipeptides with antiviral activity have not been reported yet. Furthermore, the organic acids produced by lactic acid bacteria have been reported to possess strong antimicrobial activity with the concomitant pH reduction against microbes (Matsuda, 1994; Naidu *et al.*, 1999). We investigated the quantity of each cyclic dipeptide in the extracted mixtures from the culture filtrate without organic acids, and we observed that these mixtures were the anti-influenza viral complex (Table 2). The different concentrations between the mixtures of cyclic dipeptides, including *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro), without organic acids and the single compound were as follows: In the case of the 2.0% culture filtrate without organic acids, plaque-forming assays demonstrated that the concentrations of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) were 180.3 μM and 165.6 μM in contrast to the 2.5 mM and 5.0 mM for each single molecule, respectively (Table 2). These concentrations also corresponded to decreases between 13.9–27.7 fold and 15.1–30.2 fold for the dosages corresponding to 2.5-5.0 mM *cis*-cyclo(L-Leu-L-Pro) and *cis*- cyclo(L-Phe-L-Pro), respectively. Therefore, the synergistic effect from the mixtures including these two types of cyclic dipeptides could provide clues to the identity of the antiviral substances. *Cis*-cyclo(L-Leu-L-Pro) or *cis*-cyclo(L-Phe-L-Pro) showed a drastic antiviral activity compared with that of cyclo(Met-Pro) or cyclo(Val-Pro) against the influenza A virus (Table 4). This result suggests that *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) could be regarded as active cyclic dipeptides against the influenza A virus.

The presence of double bonds at the α, β-positions in both amino acid residues of these cyclic dipeptides has been suggested to be required for antimicrobial activities, and also the NH proton in a diketopiperazine ring or the conformation (e.g., boat-up and boat-down form) of the planar structure of the pyrroline and diketopiperazine rings might have affected their inhibitory activity against microbes (Borthwick, 2012). Predictive NMR results have revealed that the majority of the energetically favorable conformers involved cyclic dipeptides that adopted a boat conformation (Li *et al.*, 2013). Nevertheless, antiviral activity has not been confirmed through the structural characteristics of the cyclic dipeptides. In addition to these aspects of the debates about the bio-active structures, the cytotoxicity should be considered. The cytotoxicity of *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) against MDCK cells suggests the possibility that they might reduce the growth rate of MDCK cells, and the cell growth was limited at high concentrations of the cyclic dipeptides (Fig. 3).

In conclusion, we searched for antiviral compounds and determined their structures by closely approaching the cyclic dipeptides from lactic acid bacteria, and we observed synergistic effects between the cyclic dipeptides from the culture filtrate without organic acids and a single cyclic dipeptide. Specific functions of cyclic dipeptides were thought to be depended on enriched chirality, and the availability of 4 positions that could alter the stereochemistry and conformational structures was believed to have a crucial role in antiviral activity. Therefore, other approaches for investigating the antiviral activity of cyclic dipeptides must focus on the relationship between antiviral function and structural verifications. Furthermore, the mechanisms of influenza viral infections have been suggested to be triggered by genes related to the repression of adrenocorticotropic hormone, which causes decreased cortisol concentrations (Jefferies *et al.*, 1998). Among the genes that are triggered during influenza viral infections, human protease genes or unidentified genes related to the cleavage of virus hemagglutinin proteins (Steinhauer, 1999) can be useful tools for revealing the basis of the infection. Therefore, investigations of the effects of cyclic dipeptides and the genes that cause viral infections may provide important clues to the infection mechanism.

The significant antiviral activity of the culture filtrate without organic acids suggests that the synergistic effect of cyclic dipeptides from fermented food materials could provide a novel direction for developing antiviral substances. Although cyclic dipeptides were effective at somewhat high concentrations in this study, these compounds are natural antibiotics

that were originated from fermented foods. Furthermore, the fractions from various lactic acid bacteria, such as *Lb. sakei* LBP-S01, *Lc. lactis* LBP-S03, *Ln. mesenteroides* LBP-K06, *Lb. plantarum* LBP-K10, *W. cibaria* LBP-K15 and *W. confusa* LBP-K16, have been thought to produce and excrete analogous metabolites (data not shown); these cognate compounds simultaneously demonstrated antiviral functions. Moreover, the compounds *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) from the culture filtrate of *Lb. plantarum* LBP-K10 that demonstrated antiviral activity against influenza A (H3N2) have been submitted in patents application (Republic of Korea Patent Application no. 10-2012-0111164, 2012; PCT/KR2013/002077, 2013).

Acknowledgements

We thank Dr. Chun Kang (Center for Infectious Diseases, National Institute of Health, Korea Centers for Disease Control and Prevention, Cheongwon-gun, Chungcheongbuk-do, South Korea) for helpful materials, including the epithelial MDCK cells and the influenza A (H3N2) virus. We also thank NCIRF at Seoul National University for supporting GC-MS experiments.

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