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Purification and Characterization of Bacteriocin J105 Produced by *Lactococcus lactis* subsp. *lactis* J105 Isolated from Kimchi

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Abstract Bacteriocin J105 is a proteinaceous inhibitory substance produced by *Lactococcus lactis* subsp. *lactis* J105 isolated from Kimchi. Bacteriocin J105 was purified to homogeneity by the pH-dependent adsorption-desorption method and reverse-phase HPLC from the culture broth of *Lactococcus lactis* subsp. *lactis* J105. Purification of bacteriocin J105 resulted in a 1.47-fold increase in the specific activity and the recovery was 1.5%. Its molecular mass measured by the electrophoretic pattern in the sodium dodecyl sulfate polyacrylamide gel was about 3.4 kDa. It was stable at 121°C for 15 min at pH between 2 and 4. However, at pH above 5, bacteriocin was rapidly inactivated. Twenty-one residues from the N-terminal portion of bacteriocin J105 were sequenced using sequence analysis of lantibiotics. Bacteriocin J105 showed significant homology with known nisin A from lactic acid bacteria.

Key words: *Lactococcus lactis* subsp. *lactis* J105, kimchi, bacteriocin, purification, lantibiotics

Lactic acid bacteria include the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Carnobacterium*, which are the main microflora of dairy, meat, and fermented foods. Lactic acid bacteria have been traditionally used for the fermentation of foods and beverages, because of their contribution to flavor and aroma development and to spoilage retardation. Recently, it has become clear that lactic acid bacteria can produce and excrete inhibitory substances, such as hydrogen peroxide, diacetyl, and bacteriocin, in addition to organic acids [2, 5].

Bacteriocins are biologically active peptides, proteins, and protein complexes produced by bacterial species acting against related species [14, 27, 31]. Lactic acid bacteria which produce bacteriocin have been isolated from dairy products, fish products, vacuum packed meat, and fermented meat and vegetables [34]. Some bacteriocins produced by lactic acid bacteria (LAB) may also inhibit the growth of pathogenic bacteria and spoilage microorganisms during food processing and food fermentation, which may be of interest to the food industry [26, 28, 30]. However, the application to the food industry has been limited due to its insolubility at a neutral pH. Therefore, it is necessary to find novel natural bacteriocins, with enough antimicrobial activity, as food preservatives [2, 5, 32].

A number of bacteriocins produced by lactic acid bacteria have been identified, but only a few have been purified and characterized [1, 13]. Nisin has been widely used as a food preservative [7, 11]. It is active against Gram-positive bacteria and is found as several types (nisin A, B, C, D, or E) that differ in both amino acid compositon and biological activity [10]. Nisin A is a 34 amino acid peptide containing lanthionine and methyllanthionine residues [9, 16]. The existence of unusual amino acids such as lanthionine or methyllanthionine residues has been shown in the structure of lactocin S [23] and lacticin 481 [29], and these have been named lantibiotics [3, 33].

Recently, bacteriocin has become important as a new kind of biopreservative or as a bioregulator of microflora in various fermented food systems [4]. Above all, screening of new bacteriocin is of great significance in the development of a start culture [30]. Kimchi is a traditionally fermented Korean food, consisting of vegetables fermented by lactic acid bacteria (LAB). The species of lactic acid bacteria in fermentation of Kimchi can influence its storage characteristics and the quality of the product. Therefore, the most important concern in the Kimchi industry is to develop an effective control method for fermentation and preservation using appropriate lactic acid bacteria as a starter. For the mass production and quality control of Kimchi, the studies on the microorganisms in Kimchi have been extensively carried out [18, 19, 38].

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We isolated bacteriocin-producing *Lactococcus lactis* subsp. *lactis* J105 from Kimchi and discovered that its bacteriocin had a broad spectrum of antimicrobial activity against Gram-positive bacteria [17]. The bacteriocin J105 produced from *Lactococcus lactis* subsp. *lactis* J105 was purified and its amino acid sequence was determined.

MATERIALS AND METHODS

Bacterial Strains and Media

A lactic acid bacteria J105 was isolated from fermented Kimchi. The strain was identified as *Lactococcus lactis* subsp. *lactis* through morphological, cultural, biochemical, and 16S rDNA sequence analysis [37]. It was cultured anaerobically in MRS (Difco) for 16 h at 30°C. The strains used as the indicator organisms for antimicrobial activities are shown in Table 2. *Lactobacillus*, *Leuconostoc*, and *Pediococcus* strains were propagated in MRS broth (Difco) for 24 h at 37°C. *Escherichia coli, Staphylococcus*, and *Bacillus* strains were propagated in Tryptic Soy Broth for 24 h at 30°C. Stock cultures were stored in broth with 50% glycerol at -70°C.

Bacteriocin Activity Assay

The activity of the bacteriocin to *Lactobacillus delbruekii* subsp. *delbruekii* was determined by a modification of the method developed by Holo *et al.* [10]. Fifty μl of fresh culture (Absorbance at 660 nm=0.1 to 0.4) was added to each dilution (50 μl) of bacteriocin extracts. After 30 min of incubation at 37°C, Eppendorf tubes were filled up to 1 ml by adding 900 μl of MRS broth, and incubated for 5 h at 37°C. The growth inhibition was evaluated by measuring absorbance at 660 nm. One bacteriocin unit (BU) was defined as the amount of bacteriocin which inhibited growth of the indicator organism by 50% (50% of the turbidity of the control culture without bacteriocin) [6].

Effect of Heat, Enzyme, and pH on Bacteriocin Activity

To determine the effect of heat on the bacteriocin activity, aliquots (1 ml) of dialyzed and neutralized bacteriocin preparation were heated at 100°C for 1 h, 110°C for 10 min, and 121°C for 15 min, and then quickly chilled with ice water. For the determination of enzyme susceptibility, dialyzed bacteriocin preparations were treated with various enzymes at a final concentration of 1 mg/ml. All enzymes (lipase, α- and β-amylase, lysozyme, protease, pepsin, trypsin, and trypsinogen) were dissolved in 0.01 M sodium phosphate buffer, pH 7.2. Bacteriocin was mixed with equal volumes of the enzyme solutions and incubated at 37°C for 2 h. As a control, bacteriocin without enzyme was treated in the same manner as the test preparations. After incubation, the activity of the treated samples was evaluated by the well diffusion test. In order to evaluate the pH stability of the bacteriocin, aliquots (5 ml) of bacteriocin preparations were adjusted to pH 2 to 10 with 5 M NaOH or HCl. After 1 day of storage at 4°C, the residual bacteriocin activity was determined.

Adsorption and Desorption Methods

For extraction of bacteriocin, the culture broth was heated at 80°C for 30 min to kill the cells and to inactivate proteolytic enzymes, and was then adjusted to pH 6.5 with 5 M NaOH [36]. The cells were harvested by centrifugation at 7,550 ×g for 20 min and washed twice in 5 mM sodium phosphate (pH 6.5). The pellets were then resuspended in 50 ml of 5 mM sodium phosphate, adjusted to pH 2.5 with 5% phosphoric acid, and then mixed with a magnetic stirrer for 24 h at 4°C to desorb the bacteriocin from cells. Cell suspensions were centrifuged at 12,000 ×g for 15 min, and the bacteriocin-containing supernatants were recovered and concentrated by vacuum centrifugation.

Reverse-Phase Chromatography

The fraction containing bacteriocin was isolated using a C_{18} Sep-pack cartridge column (Millipore Co, Milford, Calif). Two ml of the bacteriocin preparation was injected to the column and eluted using a discontinuous gradient with mobile phases A [0.1% v/v Trifluoroacetic acid (TFA)] and B (Acetonitrile in a 0.1% TFA). Active fractions were concentrated in a vacuum centrifuge, resuspended in 1 ml of mobile phase A, and analyzed by HPLC (Waters Alliance), using a 30 cm Bondapak C_{18} column (Millipore Corp., Milford, MA, U.S.A.). After equilibration of the column with solvent A at a flow rate of 1 ml/min, the bacteriocin peptides were eluted by a linear gradient from 100% solvent A to 70% solvent B in 45 min. Peptide was monitored spectrophotometrically at 254 nm. Fractions representing all peaks were collected manually in vials and assayed for bacteriocin activity. The bacteriocin samples were rechromatographed on the same column. The protein concentration was determined by the method of Lowry *et al.* [21].

SDS-PAGE

The active fractions eluted from the final HPLC were subjected to 10-25% gradient SDS-PAGE (Mighty small, Hoeffer Instruments), at a constant voltage of 100 V for 4 h [8, 35]. Molecular weight standards including markers with M_r of 1,423 to 26,625 (*BIO-RAD*) were used. A half of each gel was stained with Coomassie Brilliant Blue R-250. To detect the activity of bacteriocin on the gel, the other half of the gel was used for an *in situ* assay by the method of Kim *et al.* [12].

Modification of Bacteriocin with Unusual Amino Acid

Bacteriocin with unusual amino acids such as 2,3-didehydroalanine (Dha), 2,3-didehydro-2-aminobutyric acid (Dhb), and 2-aminobutyric acid (Aba) was modified as described by Meyer *et al.* [22]. Two nanomoles of

bacteriocin was transferred into Eppendorf tubes, dried under high vacuum, and supplemented with 15 μl of the ethanethiol-containing modification mixture. After the incubation under argon at 50°C for 1 h, the reaction mixture was acidified with acetic acid and directly applied to automated Edman degradation.

Amino Acid Sequence

The N-terminal sequence of the protein was obtained by automated Edman degradation using an Applied Biosystem Precise automatic sequencer, and the amino acid sequence was compared with the NCBI database using the Blast program.

Results and Discussion

Inhibitory Spectrum of Bacteriocin J105

The inhibitory spectrum of bacteriocin J105, the bacteriocin produced from *Lactococcus lactis* subsp. *lactis* J105 was determined by testing its efficiency against various Gram-negative and Gram-positive bacteria (Table 1), and was found to be active against various Gram-positive organisms such as *Lactobacillus* sp., *Leuconostoc* sp., *Staphylococcus aureus*, and *Bacillus subtilis* and also against some Gram-negative bacteria such as *Acetobacter aceti*, and *Pseudomonas synxantha*. However, it did not show any activity against *E. coli* or *Streptococcus mutans*. Bacteriocin J105 inhibited food-spoilage bacteria strains such as *Bacillus* and *Staphylococcus*, thereby indicating its potential as a food preservative.

Effects of Heat, Enzyme, and pH Treatment of Bacteriocin J105

Activity of bacteriocin J105 was unaffected by heat treatment at 100°C for 1 h, 110°C for 10 min, or 121°C for 15 min (Table 2). Heat stability has a practical value if bacteriocin is used as a food preservative, because many food processing procedures involve heat sterilization. Thus, bacteriocin J105 showed potential as a food preservative for pasteurized products or canned foods. The effects of various enzymes on bacteriocin activity are shown in Table 3. Trypsin, protease, and trypsinogen had no effect on bacteriocin J105; however, pepsin caused a loss of the activity. The activity of bacteriocin J105 on treatment with lipase or amylases was not inhibited. These

Table 1. Antimicrobial spectrum of bacteriocin J105 produced by *Lactococcus lactis* subsp. *lactis* J105.

Indicator strain	Antimicrobial activity*
Acetobacter aceti IFO3281	++
Bacillus subtilis ATCC21697	++
Corynebacterium glutamicum ATCC13058	+
Escherichia coli ATCC9637	-
Lactobacillus brevis IFO13109	+
Lactobacillus casei KCRZ 1121	+++
Lactobacillus delbueckii IFO3534	+++
Lactobacillus helveticus CNRZ 1096	++
Lactobacillus helveticus CNRZ 1094	+
Lactobacillus plantarum ATCC10830	+++
Leuconostoc mesenteroides ATCC10830	+++
Pediococcus acidilactici KCTC 3101	+++
Streptococcus mutans ATCC25922	-

*The radius of the inhibition zone was indicated by the following: -, 00.5 mm; +, 0.51 mm; ++, 125 mm; +++, 25-45 mm; ++++, above 45 mm.

Table 2. Heat stability of the bacteriocin J105 produced by *Lactococcus lactis* subsp. *lactis* J105.

Temperature (°C)	Treated time	Bacteriocin activity (BU/ml)
4	24 h	3831.26
100	1 h	3966.50
110	10 min	3310.96
121	15 min	3677.85

Table 3. Effect of various enzymes on the bacteriocin J105 activity of *Lactococcus lactis* subsp. *lactis* J105.

Enzyme	Antimicrobial activity on indicator strain*
Control	++
α-Amylase	++
β-Amylase	+++
Lysozyme	+++
Trypsin	++
Lipase	++
Pepsin	-
Protease	+
Trypsinogen	+

**Lactobacillus delbrueckii* subsp. *delbrueckii* was used as indicator strain and the radius of the inhibition zone was indicated by the following: -, 0-0.5 mm; +, 0.5-1 mm; ++, 1-25 mm; +++, 25-45 mm; ++++, above 45 mm.

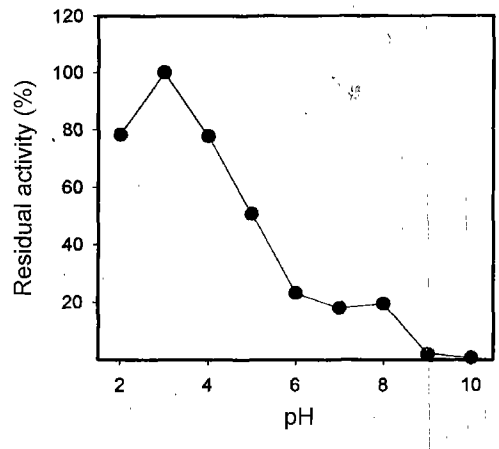

Fig. 1. pH stability of the bacteriocin J105 produced by *Lactococcus lactis* subsp. *lactis* J105.

Table 4. Purification of bacteriocin J105 from *Lactococcus lactis* subsp. *lactis* J105.

Steps	Volume (ml)	Total protein (mg)	Total activity (BU)	Specific activity (BU/mg)	Yield (%)
Adsorption-desorption soln.	4	9.80	76,000	7,755.10	100
C_{18} Sep-pack	1	0.60	6,064.24	10,107.07	7.98
RP-HPLC	0.8	0.10	1,142.77	11,427.70	1.50

results confirmed that neither lipid nor carbohydrate moieties were essential to the bacteriocin activity. Bacteriocin J105 was stable in the narrow pH range from 2 to 4 (Fig. 1). The activity was reduced at pH above 5. At pH 10, bacteriocin J105 was completely inactivated. Heat stability of bacteriocin J105 agreed with those obtained from other LAB bacteriocins [25]. The pH stability profile is in agreement with nisin and lactostrepcins, which are destabilized at high pH and netural pH [15], respectively. In addition, the result identified bacteriocin J105 as a lanthionine-containing bacteriocin such as nisin, since lanthionine and dehydroamino acid have been suggested to confer stability on the active conformation of the lantibiotic against heat, acids [16], and proteinases present in the producer cells [3].

Purification of Bacteriocin J105 Produced by *Lactococcus lactis* subsp. *lactis* J105

Bacteriocin J105 was purified by a pH-dependent adsorption-desorption method, followed by subsequent use of C_{18} Sep-pack and reverse-phase HPLC. The results of the purification are given in Table 4. The bacteriocin activity peak was detected at 53% acetonitrile in the reverse-phase column (Fig. 2a). The final recovery was 1.5% of the original activity and the specific activity was 11,427 BU/mg protein. Rechromatography of the bacteriocin-containing fraction on the reverse phase column showed one peak, indicating homogeneity of the bacteriocin (Fig. 2b).

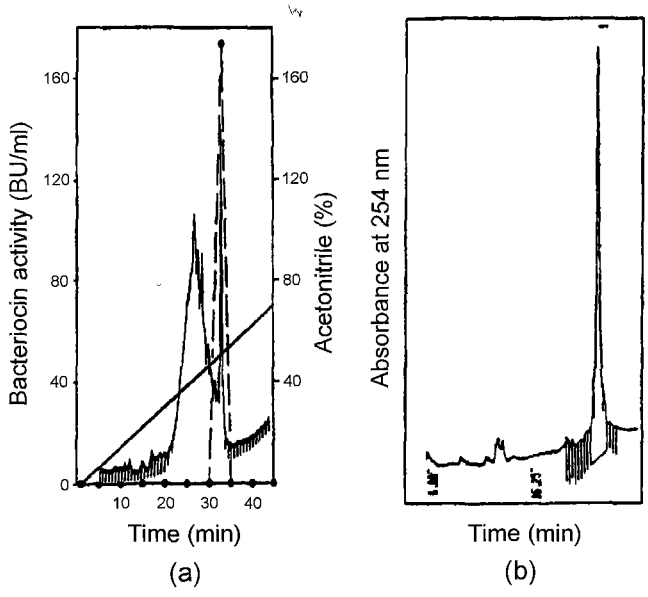

Fig. 2. Purification and identification of bacteriocin J105 by HPLC.
(a) Reverse phase chromatography on a C_{18} column of C_{18} Sep-pack sample. - -, Bacteriocin J105 activity; -, Acetontrile concentration —, Protein concentration. (b) Purity of bacteriocin J105 during rechromatography on the reverse phase HPLC.

SDS-PAGE

After purified bacteriocin J105 was concentrated, it was run on 10-25% gradient SDS-PAGE to identify the molecular weight of the protein [8, 35]. A Coomassie blue-stained SDS-PAGE gel revealed a single band with molecular weight of about 3.4 kDa. This band showed antimicrobial activity against *Lactobacillus delbrueckii* subsp. *delbrueckii* (Fig. 3), indicating that the bacteriocin J105 was a relatively small peptide, similar to nisin, lactococcin A [10], lactacin F [24], and lactocin S [23].

Amino Acid Sequence

Isoleucine was detected as the first N-terminal amino acid residue, however, the remaining amino acid sequence could not be determined by the Edman degradation method. It suggested that bacteriocin J105 contained unusual amino acids such as 2,3-didehydroalanine (Dha), 2,3-didehydro-2-aminobutyric acid (Dhb), 2-aminobutyric acid (Aba), and 3-methyllanthionine. Lantibiotics are antibiotic peptides produced via ribosomal synthesis of precursor proteins by

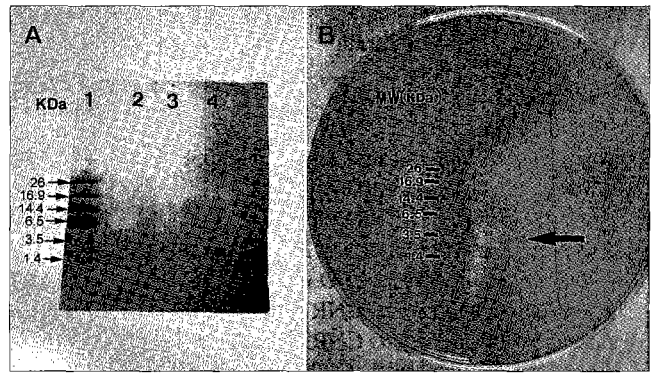

Fig. 3. Photograph of 10-25% gradient SDS-PAGE results.
Panel A: Gel was stained with Coomassie brilliant blue R-250. Lane 1, protein molecular size standards (BIO-RAD); Lanes 2-4, Purified bacteriocin. Panel B: Activity stained gel. Polyacrylamide gel was overlaid with *Lactobacillus delbrueckii* subsp. *delbrueckii* to confirm the bacteriocin band. The arrow indicates the location of the bacteriocin.

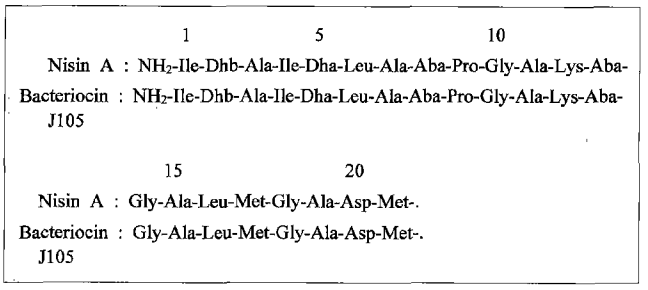

Fig. 4. Comparison of the amino acid sequence of nisin A and bacteriocin J105.
Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydro-2-aminobutyric acid; Aba, 2-aminobutyric acid.

Gram-positive bacteria. They contain various unusual post-translation modifications, including the formation of sulfide rings by lanthionine or β-methyllanthionine and 2,3-didehydroalanine (Dha). Because of these post-translational modifications, sequence analysis of lantibiotic was very difficulty. Therefore, chemical modification using an ethanethiol-containing reaction mixture was used to solve these analytical problems [22]. The chemical modifications of the unsaturated amino acids 2,3-didehydroalanine (Dha) and 2,3-didehydro-2-aminobutyric acid (Dhb) to stable S-ethylcysteinyl and β-methyl-S-ethylcysteinyl derivatives using the ethanethiol-containing reaction mixture were carried out prior to the sequence analysis by automatic Edman degradation. Lanthionine and 3-methyllanthionine bridges were also cleaved under these conditions, thereby forming stable derivatives: S-ethylcysteine and cysteine were formed from lanthionine and only S-ethylcysteine from 2,3-didehydroalanine, and 2,3-didehydro-2-aminobutyric acid was transformed to distereomers of β-methyl-S-ethylcysteine, which were identified as phenylthiohydantoin (PTH) derivatives in the form of a peak doublet eluting near the position of phenylthiohydantoin (PTH)-phenylalanine and a phenylthiohydantoin (PTH)-leucine. By using these sequence analyses of lantibiotics, the unusual amino acid sequence of bacteriocin J105 was determined. The N-terminal amino acid sequence of bacteriocin J105 was as follows: NH_2-Ile-Dhb-Ala-Ile-Dha-Leu-Ala-Aba-Pro-Gly-Ala-Lys-Aba-Gly-Ala-Leu-Met-Gly-Ala-Asp-Met-. The amino acid sequence of bacteriocin J105 was compared with that of nisin A from the NCBI gene bank and was found to have significiant homology with known nisin A, as shown in Fig. 4. However, the antimicrobial spectra of bacteriocin J105 and nisin showed some differences. Bacteriocin J105 did not show any activity against *E. coli* nor *Streptococcus mutans* (Table 1), and but nisin A inhibited certain species of *Staphylococcus* and *Streptococcus* [11]. Also, in a cell free system, 1,600 BU/ml of nisin A caused 50% inhibition of peptidoglycan synthesis of *E. coli* [20]. Nisin A was isolated from cheese, whereas bacteriocin J105 was isolated from Kimchi, a traditional Korean food, and purified and sequenced. Thus, these results suggest the possible use of bacteriocin J105 in commercial Kimchi preparation.

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