*J. Microbiol. Biotechnol.* (2002), **12**(1), 96–105

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Bacteriocin Produced by *Pediococcus* sp. in Kimchi and Its Characteristics

**KWON, DAE YOUNG, MINSEON KOO, CHAE RAN RYOO, CHANG-HOON KANG, KYUNG-HEE MIN[1], AND WANG JUNE KIM***

*Korea Food Research Institute, Songnam, Kyongki-do 463-746, Korea*
*[1]Department of Biological Sciences, Sookmyung Women's University, Seoul 140-742, Korea*

Received: August 20, 2001
Accepted: November 19, 2001

**Abstract** A bacteriocin-producing strain identified as *Pediococcus acidilactici* was isolated from kimchi. The bacteriocin was identified to belong to the pediocin family and exhibited bactericidal activity against most Gram-positive bacteria as well as some Gram-negative bacteria. The bacteriocin was stable up to 80°C with wide pH ranges (5.0–10.0). The bactericidal activity remained unchanged after treatment with nonproteolytic enzymes such as nuclease and α-amylase, however, it was destroyed after treatment with protease. The bacteriocin was effectively extracted by the pH-mediated adsorption-desorption method and purified effectively by semi-preparative RP-HPLC. The molecular weight of the bacteriocin was 4,622, as determined by electrospray mass spectrometry. The amino acid sequence consisted of 44 amino acid residues with four cysteines. The high solubility and pH stability of the isolated pediocin provide definite advantages over nisin and other bacteriocins in regards to its potential applications.

**Key words:** Bacteriocin, pediocin, *Pediococcus*, kimchi, primary structure

Bacteriocins are proteinaceous antagonistic compounds that normally inhibit the growth of closely related species, yet produce and inhibit the growth of certain pathogens and spoilage microorganisms. Therefore, a large volume of research has been focused on the use of bacteriocins as natural food preservatives [18, 22]. As a result, many kinds of bacteriocins such as nisin, sakacin, enterocin, and pediocin have been isolated from western foods, including wine, cheese, and sausages [12, 20, 34]. Their structural and functional characteristics have also been studied for application in food industries [11, 13, 16].

In oriental countries, more than 100 fermented foods are produced in each country. In particular, Korea is famous for many kinds of fermented foods, such as kimchi, doenjang, chongkukjang, jeotkal, and others. Therefore, the possibility of identifying microorganisms that produce diverse and powerful bacteriocins is higher than in western foods. Nonetheless, there have been relatively few reports about bacteriocins produced by lactic acid bacteria in oriental foods [7, 17, 23, 25].

Kimchi is one of the most famous Korean traditional fermented vegetable foods. There are more than 150 types of kimchi depending on the ingredients and preparation methods used. The most widely consumed and famous one is cabbage kimchi. The important microbes in kimchi fermentation, especially in the case of cabbage kimchi, are lactic acid bacteria such as *Leuconostoc*, *Lactobacillus*, *Streptococcus*, and *Pediococcus* [5, 28, 37]. It is already known that the microflora in kimchi have been constructed by inhibiting the resident microorganisms with a variety of antimicrobial agents, such as certain chemicals, organic compounds, or bacteriocins produced by sequential microorganisms [24]. Consequently, kimchi has been a focused source for isolating microbes that produce bacteriocins. Recently, a few papers on the production of bacteriocins from the lactic acid bacteria in kimchi have been published [5, 15, 25, 29, 34]. However, the microorganisms isolated and the bacteriocins purified were limited to certain bacteria and nisin. In addition, only a few papers have identified the structure of purified bacteriocins.

Accordingly, the current study was undertaken to screen and identify bacteriocin-producing lactic acid bacteria from cabbage kimchi, and to characterize its primary structure to elucidate the structure-function relationship of bacteriocins.

## Materials and Methods

### Materials and Bacterial Strains

The cabbage kimchi used was a commercial product purchased from a local market in Seoul (1999). The

*Corresponding author
Phone: 82-31-780-9110; Fax: 82-31-709-9876;
E-mail: wjkim@kfri.re.kr

cabbage kimchi was homogenized in a stomacher blender (Somacher 400, Seward, U.K.) with sterilized distilled water and then used to isolate the microorganisms.

In the primary screening to select the bacteriocin-like inhibitor-producing lactic acid bacteria from the kimchi, *Enterococcus faecalis* KFRI 354 supplied from Korea Food Research Institute was used as the bacteriocin-sensitive indicator strain, unless otherwise specified. The cultures were maintained in appropriate broths with 15% (v/v) sterile glycerol and stored at −70°C. Before the experiments, the cultures were propagated twice in 5 ml of an MRS broth for 18 to 24 h to make them physiologically active.

The nutrient agar, tryptic soy broth, reinforced clostridial medium, and brain heart infusion were all supplied by Difco Laboratories (Detroit, MI, U.S.A.), while the MRS broth was bought from Merck (Darmstadt, Germany).

Protease (pronase E) and nuclease were purchased from Sigma (St. Louis, MO, U.S.A.) and α-amylase of *Bacillus licheniformis* was obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of reagent and HPLC grade.

### Isolation of Bacteriocin-Producing Bacteria

The bacteriocin-producing strains were isolated using a modified sandwich method [22]. Ten grams of the cabbage kimchi were homogenized in a stomacher blender for 2 min with 90 ml of sterile distilled water. One hundred-microliter portion of this solution was then added to 5 ml of MRS with 1.5% agar, mixed well, and poured over the plate. After solidification of the plate, 5 ml of MRS with 0.75% agar (soft agar) was overlaid with 5 ml of MRS soft agar containing the indicator ($5\times10^6$ cfu/ml), *E. faecalis* KFRI 354. The plates were incubated anaerobically (GasPak; BBL, Cockeysville, MD, U.S.A.) to rule out any pseudo-effect due to hydrogen peroxide ($H_2O_2$) [19]. Those colonies exhibiting a large halo (>10 mm) were picked for further tests.

### Identification of Bacterial Strain

Conventional morphological and physiological tests were performed to identify the bacteria isolated from the kimchi. The carbohydrate fermentation patterns for the isolated bacterial strain were determined using an API CHL 50 test (API, bioMérieux, bioMérieux sa, France) as described by the manufacturers, and 16S rDNA sequence analyses were performed using a Big Dye Terminator Cycle Sequencing Kit with an automatic DNA kit from Applied Biosystems (Model 310, Perkin-Elmer, Foster City, CA, U.S.A.). The 16S rDNA sequence of the strain was aligned with the 16S rRNA gene sequence of lactic acid bacteria and certain other related taxa to construct a phylogenetic tree and compare the level of similarity.

### Antimicrobial Spectra of Bacteriocin

Using the isolated colonies, the bactericidal activities against various bacteria (see bacterial strains in Table 1) were tested using the deferred method as follows [22]: an overnight culture of the isolated strain was stabbed onto the surface of an MRS agar, and then incubated anaerobically at 37°C for 24 h to allow colony development. Approximately $5\times10^6$ cfu/ml of the test bacteria were inoculated onto 5 ml of an appropriate soft agar and poured over the plate in which the producer organism had been grown. The incubation temperatures were 26, 30, and 37°C depending on the test bacteria. After 24 h of incubation, the plates were checked for inhibition zones.

### Assay of Bactericidal Activity

The bactericidal activities of the bacteriocin for the following steps were assayed by the agar-well diffusion method with certain modifications, unless otherwise stated [42]. Forty microliter sample (cell-free extract or purified bacteriocin) was added to a 6-mm well in MRS agar plates previously inoculated with the indicator strain (*E. faecalis* KFRI 354), and allowed to diffuse for 8 h at 4°C. The plates were then incubated at 37°C for 24 h and the zones of inhibition were examined. An arbitrary unit (AU) was defined as the reciprocal of the highest dilution showing a 2-mm clear zone.

### Culture Conditions for Bacteriocin Production by Isolated Strain

**Optimization for production of bacteriocin.** Under general optimized culture conditions for lactic acid bacteria (MRS media, pH 6.5, and incubation at 37°C anaerobically) [39], the growth curve of the isolated strain was constructed using the standard plate method [2] after inoculating the cells at a rate of 1% (v/v) in a MRS broth and incubating for 24 h. The bactericidal activity of the culture against the indicator strain was investigated by an agar-well diffusion assay every two hours using cell-free extracts (see following section). Any pH changes were also recorded.

**Comparison between aerobic and anaerobic cultures.** To investigate the effect of oxygen on the production of bacteriocin during the cultivation of the isolated strain in MRS broth, the inoculated MRS broth was incubated at 37°C either aerobically or anaerobically. A glove box (Coy Laboratory Products, Ann Arbor, MI, U.S.A.) under nitrogen was used for the anaerobic conditions. The bactericidal activity of each cell-free extract was assayed after 12 h of cultivation.

### Purification of Bacteriocin

**Cell-free extract.** After cultivating the strain for 12 h (early stationary phase) in the MRS broth at 37°C anaerobically, the cell-free extract was obtained by removing the pellet by centrifugation (2,870 ×g, 5 min) and filtration through a 0.2 μm pore-size filter (Sartorius AG, Göttingen, Germany).

**Partially purified bacteriocin.** Using the cells harvested after 12 h, the concentrated bacteriocin was obtained using

the pH-mediated adsorption and desorption method [8, 10], which was previously used to purify bacteriocins like pediocin, because the isolated strain was identified as *Pediococcus acidilactici* (see results). To remove salt, the bacteriocin solution was dialyzed against distilled water using a Spectra/Por$^r$ 7 (cut-off 1,000 Da, Spectrum, Houston, TX, U.S.A.) and then lyophilized.

**Isolation of bacteriocin.** Purification of the bacteriocin by reversed phase high performance liquid chromatography was performed by the conditions described in the previous paper [10], with a slight modification.

### Characterization of Bacteriocin Produced by Isolated Strain

**Stability under heating and pH.** The bacteriocin (see mentioned above) from the isolate was heated for 30 min at 37, 50, 60, 70, and 80°C, and at 121°C for 15 min, and the residual bactericidal activity was then determined by the agar-well diffusion method. To determine the effect of pH on the bacteriocin activity, 50 µl of the cell-free extract was suspended in 150 µl of the following 1 M buffers: citric acid buffer at pH 2.0 and 5.0, acetic acid buffer at pH 5.0 and 6.0, phosphate buffer at pH 6.0 and 8.0, and Tris buffer at pH 8.0 and 10.0. The samples in each pH buffer were kept at room temperature for 30 min and then the bactericidal activity was examined. For these two experiments, partially purified bacteriocin was used due to the difficulties of purification.

**Sensitivity to enzymes:** To determine whether the bacteriocin consisted of a sole protein [3] the bacteriocin purified by HPLC was treated with various enzymes, such as protease, α-amylase, and nuclease. Ten microliters of an enzyme solution containing 5 mg of each enzyme in 1 ml of 50 mM phosphate buffer (pH 7.0) were added to 40 µl of the purified bacteriocin solution (250 µg of the bacteriocin was dissolved in 200 µl of the buffer). For α-amylase, the pH of the phosphate buffer was 6.0. After reacting with the enzymes at 37°C for 1 h, the residual activity was measured using an agar-well diffusion method against the indicator strain.

**Sensitivity of ethanol on bactericidal activity.** To investigate the effect of ethanol on the bactericidal activity due to a change in the conformational structure by ethanol [11], the purified bacteriocin was dissolved in 50% ethanol and the antimicrobial activity was assayed.

**Analysis of amino acid sequence.** The amino acid sequence of the bacteriocin was determined by Edman degradation [9] (250 picomoles for each sample) using an Applied Biosystems 491 Protein Sequencer (Perkin Elmer). The sample was loaded using a biobrene-coated fiber as a support.

**Determination of molecular weight by mass spectrometry.** The determination of the molecular weight of the isolated bacteriocin was performed using an electrospray mass spectrometer (MS) (Platform II, Micromass, Manchester, U.K.). The mass scale was calibrated using bovine serum albumin. Peptides (25 picomoles) dissolved in a water/acetonitrile (1:1, v/v) mixture containing 0.2% formic acid were introduced using a microliter syringe.

## Results and Discussion

### Identification of Bacteria in Kimchi to Produced Bacteriocin

A strain of bacteriocinogenic bacteria was isolated from naturally fermented cabbage kimchi by the sandwich method [22]. The strain exhibited bactericidal activity against 29 out of 38 strains used as indicators (Table 1). The carbohydrate fermentation profile, cellular DNA G+C content, and fatty acids composition of the cell membrane were all analyzed (data not shown). Based on sequence analysis of the 16S rDNA, a phylogenetic tree was constructed as shown in Fig. 1. The isolated strain was identified as *Pediococcus acidilactici* with 99.7% similarity. The morphological and physiological characteristics: Gram-positive coccus, nonmotile, oxidase negative, and catalase negative, were consistent with those of *P. acidilactici* as identified from the 16S rRNA data. Therefore, the isolated strain was named *Pediococcus acidilactici* K10.

In kimchi, more than 30 species belonging to different genera of lactic acid bacteria have already been reported [7, 26, 33, 36]. Among them, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Streptococcus faecalis*, *Leuconostoc mesenteroides*, and *Pediococcus pentosaceus* are known as the predominant lactic acid bacteria [20, 31, 40]. However, it has been reported that *P. acidilactici*, and *Pediococcus cerevisiae*, both facultative microorganisms, are not frequently detected in cabbage kimchi fermentation, whereas *P. pentosaceus* is often observed. The current authors found that nonpredominant bacteria like *Pediococcus* sp. rather than predominant lactic acid bacteria, such as *L. brevis*, *L. plantarum*, *L. mesenteroides*, and *P. pentosaceus*, produced more active bacteriocins during kimchi fermentation. Interestingly, *Pediococcus* sp., the microbe involved in the mid-stage of kimchi fermentation, produces a bacteriocin which is effective for inhibiting the growth of these predominant lactic acid bacteria (see Table 1). Some previous studies on the bacteriocins produced by lactic acid bacteria in kimchi, including *Enterococcus faecium*, *P. pentosaceus*, *L. mesenteroides*, *Leuconostoc paramesenteroides*, *Lactococcus lactis*, and *L. brevis*, have already been reported [7, 25]. However, the current study was the first to report that *P. acidilactici* was isolated as a strain producing an active bacteriocin in kimchi. In fermented sausages, the same species, *P. acidilactici* H and M, have also been isolated as strains producing bacteriocin [21, 35].

### Antimicrobial Spectrum

The *P. acidilactici* K10 isolated from kimchi exhibited a wide range of inhibitory activity against Gram-positive and

**Table 1.** Inhibitory spectrum of bacteriocin-producing strain isolated from kimchi against various bacteria determined using deferred assay [21].

| Species tested | Growth conditions | | Inhibition[a] |
|---|---|---|---|
| | Media | Temp (°C) | |
| Gram-positive bacteria | | | |
| *Lactobacillus* | | | |
| *acidophilus* KFRI 507 | MRS | 37 | ++ |
| *bulgaricus* KFRI 425 | MRS | 37 | +++ |
| *brevis* KFRI 353 | MRS | 30 | - |
| *casei* KFRI 196 | MRS | 37 | ++ |
| *confusus* KFRI 653 | MRS | 30 | ++ |
| *curvatus* KFRI 166 | MRS | 37 | ++ |
| *delbrueckii* KFRI 149 | MRS | 37 | ++ |
| *fermentum* KFRI 164 | MRS | 37 | ++ |
| *gasseri* KFRI 658 | MRS | 37 | - |
| *helveticus* KFRI 659 | MRS | 37 | - |
| *pentosus* KFRI 481 | MRS | 37 | - |
| *plantarum* KFRI 464 | MRS | 37 | - |
| *plantarum* NCDO 955 | MRS | 37 | ++ |
| *sake* KFRI 816 | MRS | 30 | ++ |
| *Pediococcus* | | | |
| *pentosaceus* KFRI 167 | MRS | 37 | + |
| *acidilactici* KFRI 443 | MRS | 37 | + |
| *crevisiae* KFRI 438 | MRS | 37 | ++ |
| *Propionibacterium* | | | |
| *freudenreichii* KFRI 668 | YGB | 30 | ++ |
| *acnes* ATCC 6919 | YGB | 30 | ++ |
| *Bacillus* | | | |
| *cereus* KFRI 437 | TSB | 30 | - |
| *coagulans* KFRI 841 | TSB | 37 | + |
| *Enterococcus* | | | |
| *faecalis* KFRI 354 | MRS | 37 | ++ |
| *faecalis* var. *liquefaciens* KFRI 675 | MRS | 37 | +++ |
| *Streptococcus* | | | |
| *agalactiae* KFRI 885 | TSB | 37 | ++ |
| *mutans* KFRI 1171 | BHI | 37 | ++ |
| *Micrococcus luteus* KFRI 454 | NA | 30 | ++ |
| *Clostridium perfringens* KFRI 752 | RCM | 37 | - |
| *Listeria monocytogenes* KFRI 799 | BHI | 37 | ++ |
| *Staphylococcus aureus* KFRI 219 | TSB | 37 | + |
| *Lactococcus diacetylactis* KFRI 185 | MRS | 37 | ++ |
| *Leuconostoc mesenteroides* KFRI 817 | MRS | 30 | ++ |
| Gram-negative bacteria | | | |
| *Pseudomonas* | | | |
| *aeruginosa* KFRI 252 | NA | 37 | + |
| *fluorescens* KFRI 194 | NA | 26 | + |
| *fragi* KFRI 462 | TSB | 30 | - |
| *Aeromonas hydrophila* KFRI 461 | NA | 30 | + |
| *Shigella flexneri* KFRI 445 | NA | 37 | + |
| *Salmonella typhimurium* KFRI 191 | NA | 37 | - |
| *Escherichia coli* KFRI 272 | NA | 37 | - |

Abbreviations: KFRI, Korea Food Research Institute (Songnam, Korea); NCDO, National Collection of Food Bacteria (Reading, U.K.); ATCC, American Type Culture Collection (Rockvile, MD, U.S.A.); NA, nutrient agar; TSB, tryptic soy broth; RCM, reinforced clostridial medium; BHI, brain heart infusion; YGB, yeast glucose broth.

[a] -, absence of inhibition zone; +, 1 to 10 mm; ++, 11 to 20 mm; +++, 21 mm and over (diameter of inhibition zone).

negative bacteria (Table 1). The inhibition of Gram-positive bacteria by *P. acidilactici* was not unexpected. However, some Gram-negative bacteria, such as *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Shigella flexneri* were also inhibited. Normally, Gram-negative bacteria are resistant to bacteriocins [1].

This result is in agreement with previous reports that certain lactic bacteriocins can inhibit a limited number of

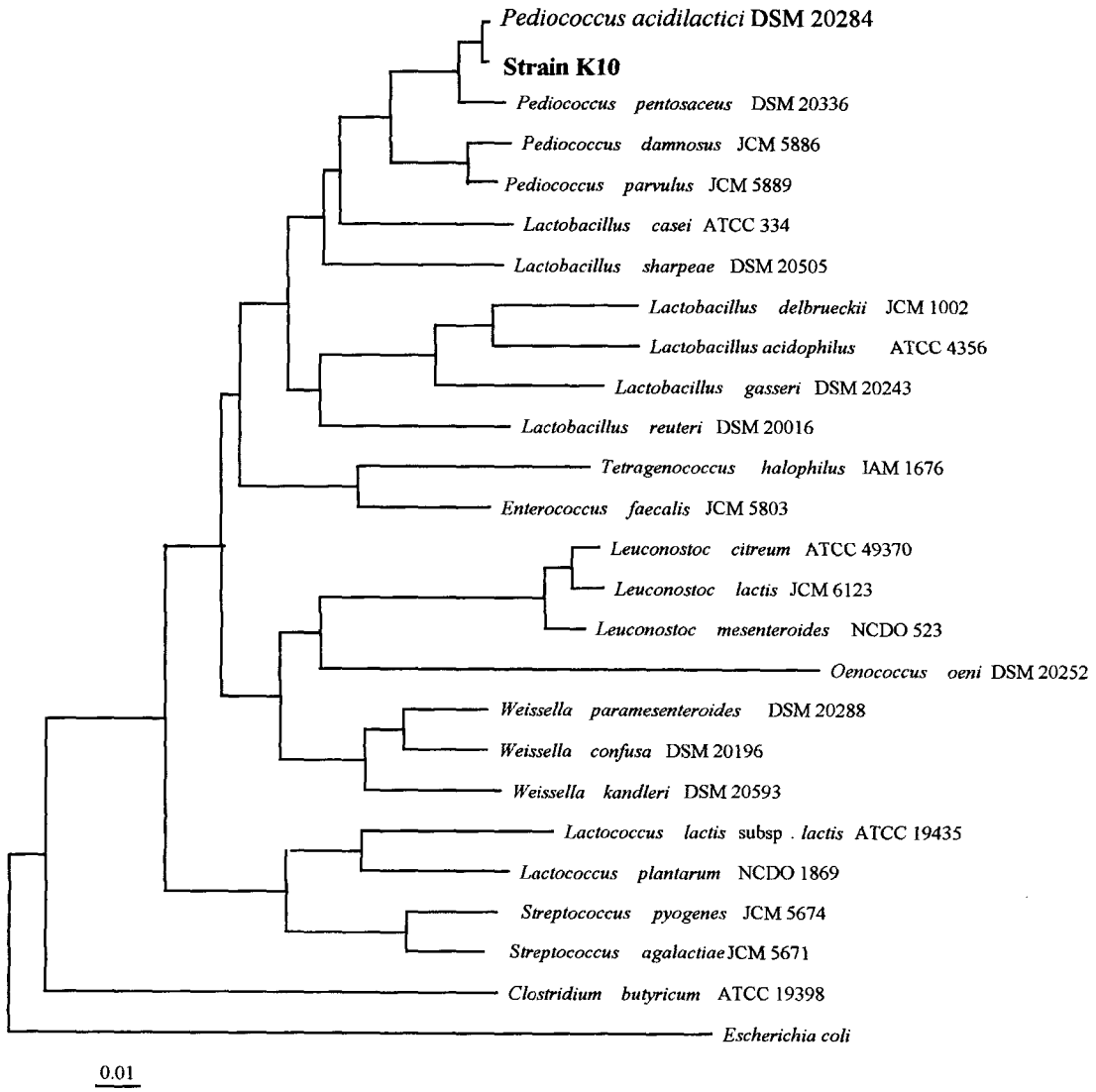


**Fig. 1.** Phylogenetic tree based on 16s rDNA sequences showing positions of strain isolated from kimchi.
Scale bar under the figure represents 0.01 substitution per nucleotide position.

Gram-negative bacteria [3, 10, 36]. A previous paper [10] also reported that pediocin AcM from *P. acidilactici* M, isolated from sausage, inhibited the Gram-negative bacterium, *A. hydrophila*, however, no bactericidal activity was detected with *P. aeruginosa, P. fluorescens*, and *S. flexneri*. Pediocin AcM also has a wider antimicrobial spectrum than pediocin PA-1/AcH, produced by *P. acidilactici* from fermented sausage [10, 16].

Of particular interest was the fact that infection-causing bacteria such as *Streptococcus agalactiae*, *Streptococcus mutans*, *Enterococcus faecalis* var. *liquefaciens*, and *Propionibacterium acnes* were also inhibited by the bacteriocin isolated from *P. acidilactici* K10. *S. agalactiae*, *S mutans*, and *E. faecalis* var. *liquefaciens* are known to cause mastitis in cows and *P. acnes* can incite or contribute to the condition known as acne. As such, *P. acidilactici* K10 has replaced the use of antibiotics in cattle farming and cosmetic production. The pathogenic Gram-negative bacteria, *A. hydrophila*, was also sensitized by *P. acidilactici* K10, as in the case of pediocin AcM [10].

## Optimization of Production of Bacteriocin

**Time course of cell growth and bacteriocin production.** To investigate the relationship between bacteriocin production and cell growth, the time course of the cell growth and bacteriocin production was studied. Therefore, the isolated strain was cultivated at 37°C in an MRS broth anaerobically. As shown in Fig. 2, the bacteriocin production by *P. acidilactici* K10 started in the late exponential phase during fermentation and reached a maximum in the early stationary phase. The maximal bacteriocin activity (133.3 AU/ml) was reached after 12 h of incubation and the pH of

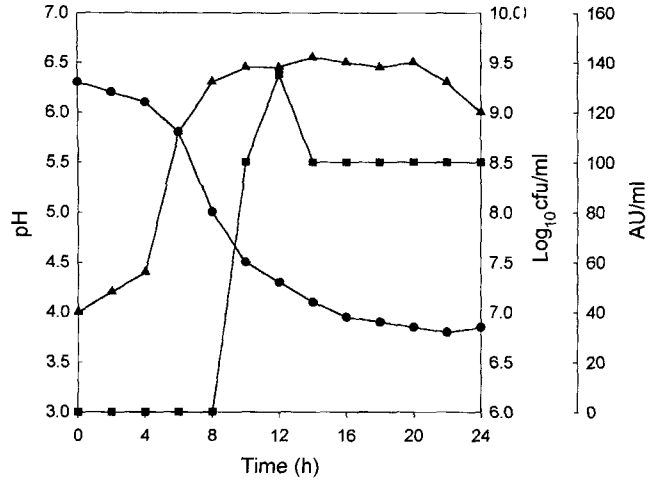


**Fig. 2.** Changes in pH (●), cell population (cfu/ml, ▲), and bactericidal activity (AU/ml, ■) of the isolated strain in MRS broth during anaerobic incubation for 24 h at 37°C.

the culture was 4.3. Therefore, the optimal incubation time for bacteriocin production from *P. acidilactici* K10 was determined to be 12 h. Generally, bacteriocins including plantaricin F and pediocins are produced in the early stationary phase [14, 41].

**Effect of oxygen on bacteriocin production.** Table 2 shows the effect of oxygen in the cultivation on the production of bacteriocin. The bacteriocin production under anaerobic conditions was higher than that under aerobic conditions. Usually, the microaerophiles, such as *Pediococcus* sp., prefer the anaerobic to aerobic condition for growth. Although *P. acidilactici* isolated from kimchi grew slowly in aerobic conditions, a substantial amount of *P. acidilactici* cell was harvested even in aerobic condition at 12 h culture. This is consistent with other reports where bacteriocin production by *P. acidilactici* under anaerobic condition was also found to be more rapid than under aerobic condition [18].

**Table 2.** Presence and/or absence of bacteriocin activity between aerobic and anaerobic culture conditions for cell-free extracts[a,b].

| Dilution rate of supernatant (fold) | Bactericidal activity | |
|---|---|---|
| | Aerobic | Anaerobic |
| 1 | +[c] | + |
| 2 | - | + |
| 4 | - | + |
| 8 | - | + |
| 16 | - | - |
| 32 | - | - |

[a]The cell-free extract was obtained by removing the pellet by filtration through a 0.2 μm pore-size filter after centrifugation.
[b]Bacteriocin activity assayed by the agar-well diffusion method against *E. faecalis* KFRI 354.
[c]+; presence of bacteriocin activity, -; absence of bacteriocin activity.

## Purification of Bacteriocin

Since the bacteriocin-producing strain was identified as *P. acidilactici*, the bacteriocin was purified using the pH-mediated adsorption-desorption method, and RP-HPLC, based on a prior study regarding the mode of action of pediocin adsorption and desorption onto/from the bacterial cell wall [4, 10]. The RP-HPLC chromatogram (Fig. 3a) showed that the partially purified bacteriocin precipitated by pH mediation was separated into five major peaks on HPLC (peaks A, B, C, D, and E). Among the peaks, peak E was identified as a bacteriocin by analyzing their bactericidal activities against the indicator strain (Fig. 4). This partially purified peak E was collected and further purified by semi-preparative HPLC to a single peak (Fig. 3b). About 1 mg of the purified bacteriocin was obtained from 1.8 l culture broth, and Table 3 summarizes the

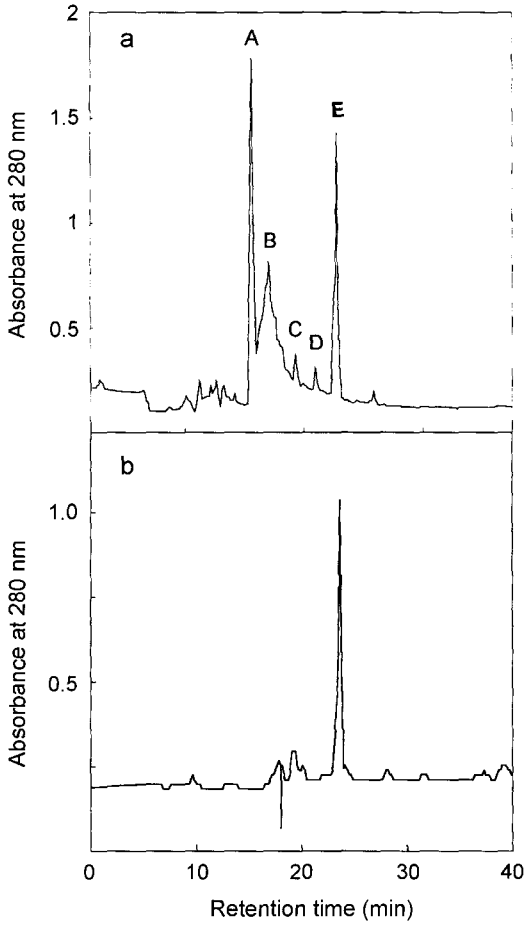


**Fig. 3.** RP-HPLC chromatograms of bacteriocin purified from *P. acidilactici* for partially purified bacteriocin by pH-mediated absorption-desorption from strain isolated from kimchi (a) and purified bacteriocin by semi-preparative RP-HPLC (b).
For purification of bacteriocin by RP-HPLC, using solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile) at a flow rate of 1.5 ml/min, the gradient was as follows: from initial to 5 min, 75% solvent A and 25% solvent B; from 5 min to 35 min, 45% solvent A and 55% solvent B.

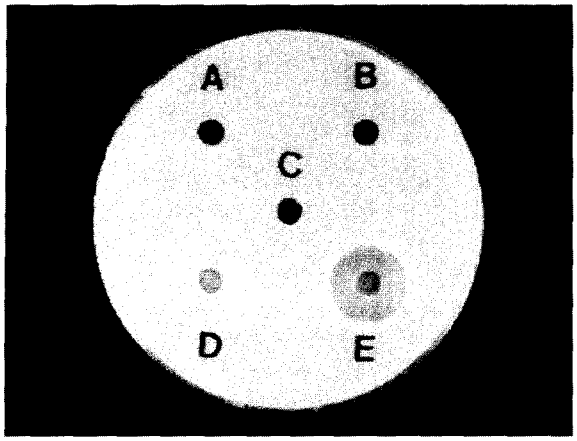


**Fig. 4.** Bioassay plate of peaks collected from RP-HPLC analysis for partial-purified bacteriocin to detect the bactericidal peak among peaks A, B, C, D, and E in Fig. 3a.
A bioassay of each peak was performed against *E. faecalis* KFRI 354 using the agar-well diffusion method [42], with 60 μl of the test solution in water per well.

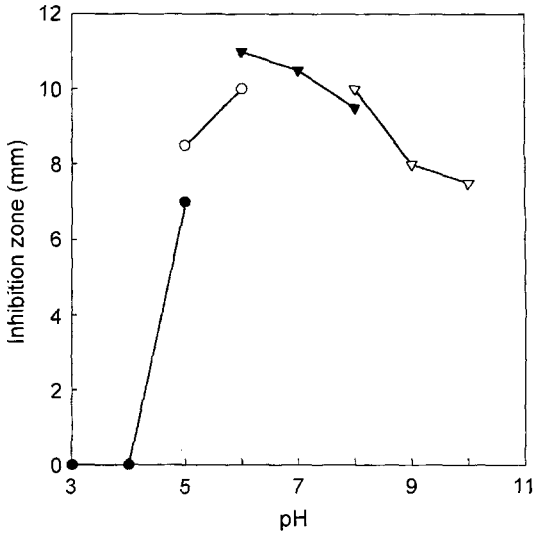


**Fig. 5.** Effect of pH on antimicrobial activity of bacteriocin from *P. acidilactici* in kimchi.
Citric acid buffer at pH 2.0 to 5.0; acetic acid buffer at pH 5.0 and 6.0; phosphate buffer at pH 6.0 to 8.0; Tris buffer at pH 8.0 to 10.0. The cell-free extracts were kept for 30 min in each buffer, then the bactericidal activity was measured by an agar-well diffusion assay against the indicator.

purification steps. The specific activity was increased 1,700-fold (Table 3).

## Characterization of Bacteriocin Activity

**Effects of pH and heat.** The effect of pH on the bactericidal activity of the bacteriocin is shown in Fig. 5. At pHs ranging from pH 5.0 to 10.0, the bacteriocin (partially purified by pH mediated adsorption-desorption) maintained its activity, whereas it lost its activity at acidic pH (<5). The bacteriocin (later identified as pediocin) from kimchi was stable up to 80°C, but lost its bactericidal activity with treatment at 121°C for 15 min (Table 4).

These results are not consistent with previous data [10], where the pediocin purified from *Pediococcus acidilactici* M by HPLC was stable through a wider range of pHs (pH 2–12), and the bactericidal activity remained even after high temperature treatment (121°C for 15 min). This indicated that the partially purified pediocin was less stable than highly pure pediocin.

In previous studies [10, 11], the current authors reported that purified pediocin was very stable at an extreme pH or high temperature due to its random coiled structure. The structure of pediocin in an aqueous solution has been revealed as a random coil [6, 11] (also see Fig. 7c). In a random coil, the denatured structure of the protein is also a random coil even under such drastic conditions as high temperature and extreme pH. Accordingly, such a structure has been suggested to be the reason why pediocin remains its activity at such drastic pHs and temperatures. In contrast, the results from the current study and other reports [4, 20] showed that pediocin was not as stable as the HPLC purified pediocin [10]. This may have been due to the purity of the bacteriocin. Sometimes, impurity or other contaminating protein in partially purified protein can be effective, and at other times mal-effective in maintaining the protein structure [26]. In the current study, impurities in the partially purified bacteriocin might have had a negative effect on the bactericidal activity, when compared to the purified bacteriocin [10].

The pediocin was found to be very stable and soluble in a wide range of pHs investigated. Thus, this high solubility and pH stability can be advantageous over nisin or other bacteriocins in food processing and preservative applications [16].

**Enzyme treatments.** The bacteriocin activity was completely lost when treated with protease (pronase E)

**Table 3.** Purification of bacteriocin from *P. acidilactici* isolated from kimchi.

| Purification stage | Volume (ml) | Protein[a] (mg/ml) | Activity (AU/ml)[b] | Total activity (AU) | Specific activity (AU/mg) | Purification (fold) |
|---|---|---|---|---|---|---|
| Cell-free extract | 1,800 | 35.46 | 133 | 239,940 | 3.8 | 0,001 |
| pH-mediated absorption-desorption | 40 | 0.34 | 400 | 16,000 | 1,176.4 | 310 |
| RP-HPLC | 1 | 0.12 | 800 | 800 | 6,666.7 | 1,754 |

[a]Determined by BCA micro-assay method [38].
[b]Activity is expressed in arbitrary units as determined by an agar-well diffusion assay against *E. faecalis* KFRI 354.

**Table 4.** Effect of heat on the antimicrobial activity of partial-purified bacteriocin from *P. acidilactici* isolated from kimchi[a].

| Temperature (°C)[b] | Inhibition zone (mm)[c] |
|---|---|
| Control | 13.5 |
| 37 | 14.0 |
| 50 | 14.0 |
| 60 | 13.5 |
| 70 | 13.0 |
| 80 | 13.0 |
| 121 | 0 |

[a]The bacteriocin activity was measured by agar-well diffusion assay against *E. faecalis* KFRI 354 [42].
[b]The partial-purified bacteriocin purified by the pH-mediated absorption and desorption method was heated for 30 min at 37, 50, 60, 70, and 80°C, and at 121°C for 15 min.
[c]The inhibition zone does not contain the well diameter (6 mm) that was added to each sample (60 µl). This is the same in Tables 5 and 6.

at pH 7.0, but was unaffected by α-amylase and nuclease treatments (Table 5). This suggests that the bacteriocin was composed of only protein, with no nucleic acid and carbohydrate moiety. Accordingly, the antimicrobial agent produced from the isolated strain (*P. acidilactici* K10) from kimchi was classified as a true bacteriocin [24].

**Molecular weight determination and amino acid sequence analysis.** The molecular weight of the bacteriocin from *P. acidilactici* K10 was 4,622, as determined by MS (Fig. 6). The mass spectrum also showed that the purified bacteriocin was a highly pure, single compound. Therefore, these results indicated that the purified bacteriocin from kimchi was a pediocin with a molecular weight of 4,626.

The amino acid sequence analysis of the pediocin showed that the bacteriocin of *P. acidilactici* isolated from kimchi consisted of 44 amino acids with 4 cysteines at positions 9, 14, 24, and 44 (Fig. 7a). The sequence of the bacteriocin from *P. acidilactici* K10 from kimchi was identical with that of the pediocins (Fig. 7b) from *P. acidilactici* strains isolated from different sources [3, 21, 35]. The molecular weight of 4,622 determined by MS was smaller than the calculated molecular weight of 4,628. This might have been due to a slight difference in the MS

**Table 5.** Sensitivity of enzyme-treated bacteriocin[a] from *P. acidilactici* from isolated kimchi.

| Treatment | Inhibition zone (mm)[b] |
|---|---|
| Control | 10.0 |
| Protease (Pronase E) | 0 |
| Nuclease | 10.0 |
| α-Amylase | 10.0 |

[a]The partially purified bacteriocin (same as in Table 4) was treated with three enzymes. The enzyme solution (50 mg in 1 ml of 50 mM phosphate buffer, pH 7.0) was added to the bacteriocin-containing buffer (250 µg in 250 µl of phosphate buffer, pH 7.0) except for α-amylase. The pH of the buffer for α-amylase was 6.0.
[b]After reacting with the enzymes for 1 h at 37°C, the residual activity was assayed using the agar-well diffusion method against the indicator [42].

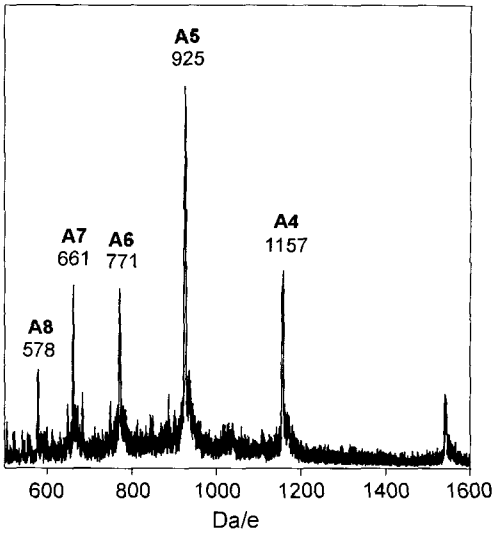


**Fig. 6.** Mass spectra of bacteriocin produced from *P. acidilactici* isolated from kimchi.

calibration and resolution. In a previous work, the molecular weight for the same pediocin was estimated as 4,626 due to one disulfide bond and determined as 4,618 by MS [10].

Assignments of the disulfide bond for the four free thiol groups (Cys 9, Cys 14, Cys 24, and Cys 44) were not performed. However, cysteines 9 and 14 must be linked by a disulfide bond, as in the case of pediocin AcM [10]. Further research is necessary to assign the disulfide bonds,

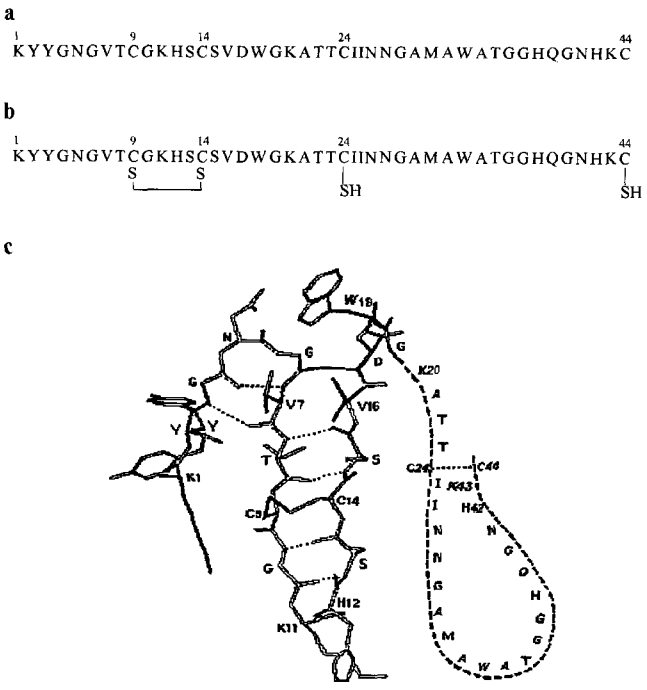


**Fig. 7.** Amino acid sequence of the pediocin of *P. acidilactici* isolated from kimchi (a) and sausage (b) [13], and the tertiary structure of pediocin PA-1 as predicted by Chen *et al.* [6].

**Table 6.** Effect of ethanol on the activity of bacteriocin from *P. acidilactici* isolated from kimchi.

| Treatment[a] | Inhibition zone (mm) |
|---|---|
| Purified bacteriocin:water (=1:1) | 7.0 |
| Purified bacteriocin:ethanol (=1:1) | 7.0 |
| Water:ethanol (=1:1)[b] | 0 |

[a]Each bacteriocin purified by HPLC was dissolved in water and ethanol and the bactericidal activity assayed by the agar-well diffusion method [42].
[b]Fifty percent ethanol without the purified bacteriocin was also assayed for its bactericidal activity, using the previous method.

especially the disulfide bond of Cys 24 and Cys 44, because the disulfide bond between these two cysteines is still controversial [11, 16, 32].

**Effect of ethanol.** According to a previous work [11], the conformation of pediocin is shifted from a random coil (see Fig. 7c) to a helical coil [11] by ethanol. A circular dichroism (CD) experiment showed that a substantial helix structure for the pediocin was observed in 50% trifluoroethanol solution [11]. The results in Table 6 show that the bactericidal activity was unaffected by ethanol. When the pediocin was dissolved in 50% ethanol, the bacteriocin activity was reduced by about 25% or less. An ethanol solution (50%) without pediocin did not create any inhibition zone against the indicator. This suggests that the conformation of the pediocin was not important in inhibiting the growth of foreign cells. The same results were observed with pediocin AcM from sausage [11].

In conclusion, the bacteriocin isolated from *P. acidilactici* in kimchi was very effective in inhibiting the related species, stable at a wide range of pHs and high temperatures, and identified as pediocin. Pediocin is widely distributed in both western and oriental fermented foods. The isolated pediocin was found to be soluble at all the pHs tested, thus this high solubility and pH stability gives definite advantages over nisin and other bacteriocins for potential applications [18]. Furthermore, the high temperature stability is very practical if pediocin is to be used as a food preservative, since many processing procedures involve heating. Finally, an analysis of the primary structure should be carried out as a key step in elucidating the structure and function relationship of pediocin.

## Acknowledgment

This work was supported in part by grant (2000-2-211-002-4) from KOSEF.

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