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ORIGINAL ARTICLE

Partial characteristics and antimicrobial mode of pediocin produced by *Pediococcus acidilactici* PA003

Jing Wang · Li Li · Xin Zhao · Zhijiang Zhou

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Abstract Pediocin was found to have an effective antimicrobial function against *Listeria monocytogenes* and some other Gram-positive bacteria produced by *Pediococcus acidilactici* PA003, which remained active between pH 2.0 and 12.0 after a 2 h treatment, at 4 °C and −20 °C for 1 month and at 40, 60, 80, 100, and 121 °C for 1 h. Furthermore, complete inactivation or significant reduction of antimicrobial activity was observed after treatment of the cell-free supernatant with proteinase K, pepsin, trypsin, and papain. According to the study on the correlation between pediocin protein secondary structure and antimicrobial activity by circular dichroism spectra analysis, as pediocin α-helix levels increased, β-strand and β-turn content decreased, and the pediocin activity decreased thereupon. Analysis of pediocin adsorption characteristics indicated that the adsorption effect of pediocin was not affected by temperature and duration, but pH was a crucial factor. In general, 98 % of the pediocin was adsorbed at pH values near 6.0, and the lowest adsorption took place at pH levels below 2.0 or above 10.0. By testing the ultraviolet absorption material and by determining the pH gradient and the membrane potential gradient, we determined that the occurrence of holes on the sensitive bacteria cell membrane, the dissolution of intracellular nucleic acid and protein material, and the dissipation of proton motive force led to sensitive bacteria cell death after treatment with pediocin.

Keywords Pediocin · Characteristics · Antimicrobial mode

J. Wang · L. Li · X. Zhao · Z. Zhou
School of Chemical Engineering and Technology, Tianjin University, No. 92 Weijin Road, Nankai District, Tianjin, People's Republic of China 300072

Z. Zhou (✉)
Microbiology Laboratory, Department of Food Science, Tianjin University, Tianjin, People's Republic of China
e-mail: zzj@tju.edu.cn

Introduction

Lactic acid bacteria are well known for their production of peptides and proteins with antimicrobial properties (i.e., bacteriocins). Bacteriocins are biologically active proteins that have an antibacterial function against Gram-positive bacterial species related to the producer strain. Some bacteriocins have a very narrow spectrum of activity, but others have a relatively broad spectrum of antibacterial activity (Marugg 1991; Barefoot and Nettles 1993; Papagianni 2003).

Much attention is being directed at food preservation by using bacteriocins because of their multiple consumer-friendly characteristics, including flavourlessness and rapid digestion of food by proteases in the digestive tract. So far, only nisin produced by *Lactococcus lactis* is a commercial product and approved food additive in most major food-producing countries. Another bacteriocin that attracts interest in the field of research and will likely be used in the food industry is pediocin (Ray 1992; Turcotte et al. 2004).

Pediocins produced by *Pediococcus* strains [GRAS (generally recognized as safe) organisms] have a wide inhibitory spectrum of activity against Gram-positive bacteria, including both spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Clostridium perfringens* (Abriouel et al. 2001). One of the most important characteristics of pediocin is its high antimicrobial activity against *Listeria monocytogenes* (Bennik et al. 1999; Guyonnet et al. 2000; Kheadr et al. 2010). *Listeria monocytogenes* is a ubiquitous pathogen in the environment, capable of producing both human and animal infections (Siegman-Igra et al. 2002).

Pediocin AcH or PA-1, of 44 amino acids, was the first and most thoroughly characterized bacteriocin among Class IIa bacteriocins. PA-1 is produced by several *Pediococcus acidilactici* strains found in meat (Nieto Lozano et al. 1992; Cintas et al. 1995; Anastasiadou et al. 2008), two vegetables

associated with *Pediococcus parvulus* (Bennik et al. 1997a), and a *Lactobacillus plantarum* strain isolated from cheese (Loesner et al. 2003). Pediocin peptides contain a consensus sequence in the N terminus (Tyr-Gly-Asn-Gly-Val-Xaa-Cys), a small hydrophobic region, and disulfide bridges, all of which are shown to be essential for bacteriocin activity (Klaenhammer 1993). Class IIa bacteriocins are currently thought to act primarily by permeabilizing the target membrane through the formation of pores. These pores are hypothesized to cause leakage of ions and inorganic phosphates, subsequently dissipating the proton motive force (PMF) (Bruno and Montville 1993; Maftah et al. 1993; Bennik et al. 1997b).

Chen and Montville (1995) reported that pediocin PA-1/AcH acts on sensitive strains through the dissipation of the PMF, a mode of action resulting in an efflux of intracellular amino acids, potassium ions, and inorganic phosphates, but not adenosine triphosphate (ATP).

However, the mode of action of pediocin in targeting bacterial cells is far from clear. In this study, pediocins were produced from a *Pediococcus acidilactici* strain of a Chinese sauerkraut origin. The main physical and chemical properties of the pediocins, including the secondary structures, were determined. The membrane-based antimicrobial action of pediocin on *Listeria monocytogenes* cells was studied to explore the mechanism of the bacteriocin.

Materials and methods

Bacterial strains

Pediococcus acidilactici PA003, a wild pediocin PA-1-producing strain, was isolated from the fermentation liquor of Chinese sauerkraut in our previous study (Zhou et al. 2006). The strain was grown in MRS broth. The indicator strain used in the pediocin assay was *Listeria monocytogenes* CVCC 1595 (purchased from the China Institute of Veterinary Drug Control) and was grown in broth medium. *Lactobacillus plantarum* CICC 6043 and *Escherichia coli* DH5α (purchased from the China Center of Industrial Culture Collection) were grown in MRS and LB broth, respectively. All other chemicals used were of analytical grade unless otherwise stated.

Determination of pediocin activity

Pediocin activity was determined by using the Oxford cup method assay (Lv et al. 2003). The antimicrobial activity unit of the pediocin was defined as the reciprocal of the highest dilution that shows inhibition of the indicator lawn and was expressed in arbitrary units (AU) per mL. The antimicrobial activity unit of the pediocin was determined by using the double dilution method (Tramer and Fowler 1964). AU/mL = $2^n \times (1000/x)$ (n, the number of wells with inhibited growth; x, bacteriocin volume in each well). Sample pH was adjusted to 6.0 with 1 mol/L NaOH.

Purification and purity analysis of the pediocin

Pediocin was purified by using the method of Yang et al. (1992). Cultures of *Pediococcus acidilactici* were treated with a water bath at 70 °C for 30 min and were afterwards cooled to room temperature for 15 min. Subsequently, the pH of the samples was adjusted to 6.0 with 1 mol/L NaOH, mixed at 25 °C for 30 min and centrifuged (8,000 rpm) at 4 °C for 30 min. Cells were washed twice with phosphate buffer solution (PBS, 5 mmol/L, pH 6.0). The precipitate was collected by centrifugation and was suspended in NaCl solution (10 mL, 100 mmol/L, pH2.0). The suspension was mixed at 4 °C for 12 h. The supernatant was filtered with a sterile 0.22 μm membrane.

Tricine-SDS-PAGE was used to analyse the purity of the purified pediocin (Sambrook and Russell 1996). Electrophoresis was conducted at a constant voltage of 60 V until the tracking dye reached the bottom of the gel. The gel was stained with Coomassie brilliant blue-R-250, and the molecular weight was estimated by using the gel documentation system Alpha-Innotech, using low molecular weight marker proteins for comparison.

The physical and chemical properties of pediocin

Purified pediocin (pH 6.0, 640 AU/mL) was exposed to heat treatments at 20, 40, 60, 80, 100, and 121 °C for 1 h. The samples were then tested for antimicrobial activity. Purified pediocin was also stored for 4 weeks at −20 and 4 °C, and the pediocin activity was assayed at 1-week intervals. The pH of the purified pediocin was adjusted to the pH range of 1.0 to 14.0 (at increments of 1.0 pH unit) by using 1 mol/L HCl or 1 mol/L NaOH solutions. Following incubation at 25 °C for 2 h, the samples were neutralized to a pH of 6.0 and were tested for antimicrobial activity. With the concentration of 0.03, 0.1, and 0.3 mg/mL pediocin (protein concentration of 640 AU/mL of purified bacteriocin was 0.1 mg/mL) was treated by the method mentioned above. Protein concentration was measured using the Coomassie brilliant blue method (Li et al. 1994). Resistance to proteolytic enzymes was determined by incubating samples of the purified pediocin in the presence of trypsin and proteinase K (0.5 M sodium phosphate buffer, pH 7.0) and pepsin and papain (0.2 M citric acid buffer, pH 2.0) at 37 °C for 3 h. All enzymes used were at the final concentration of 0.5 mg/mL. Purified pediocin in buffer was used as a control. Following incubation, enzymes were inactivated by heating for 3 min at 100 °C, and samples were examined for antimicrobial activity.

The secondary structure of pediocin

The secondary structure of pediocin was estimated from circular dichroism (CD) spectra analysis (Shen et al. 2004). This method was conducted on a Jasco J-810 spectrometer. The measured spectra were smoothed by CD with software, and the data were processed and analysed by DichroWeb online (Whitmore and Wallace 2008).

Secondary pediocin structures in different pH (2.0, 4.0, 6.0, 7.0, 8.0, 10.0, and 12.0) solutions were analysed by CD spectra analysis. Prior to analysis, pediocin was heated in a 100 °C water bath for 15 min.

Indicator cell adsorption properties of pediocin

Listeria monocytogenes CVCC 1595 was grown at 37 °C in broth medium (O.D. 600 nm≈0.7), and the cells were collected by centrifugation (12,000 rpm, 25 °C, 10 min). Pellets were washed twice with PBS (5 mmol/L, pH 6.0) and were added to the original volume of 5 mmol/L PBS. A 1-mL portion of the suspension was mixed with 0.25 mL of pediocin (640 AU/mL), and this mixture was incubated at 25 °C for 15 min. The control consisted of 1 mL of sterile PBS instead of cell suspension.

Percentage of pediocin adsorbed=100×[1−(AU/mL in cell-free supernatant - AU/mL in control I)] / (AU/mL in control II) (Yang et al. 1992).

The effect of temperature on the percentage of pediocin adsorbed was tested by incubating the experimental group and the control group at −20, 0, 4, 25, 50, 70, 90, and 100 °C for 15 min. The percentage of pediocin adsorbed by the experimental group and control group was also tested after 1, 5, 15, and 30 min at 25 °C. The cells were resuspended in PBS of different pH values (2.0, 4.0, 6.0, 7.0, 8.0, 10.0, and 12.0). Subsequently, pediocin was added and the cells were incubated at 25 °C for 15 min. Then, the percentage of bacteriocin adsorbed was tested. The influence of the enzymes was determined by incubating *Listeria monocytogenes* CVCC 1595 cells in the presence of lysozyme (Tris-HCI, pH 4.5), trypsin, pepsin, proteinase K, and papain (5 mmol/L PBS, pH 5.5) at 25 °C for 15 min. Cells were then washed by PBS (5 mmol/L, pH 6.0) four times. All enzymes were used at a final concentration of 2 mg/mL. The cells in buffers without enzyme were used as controls. Following incubation, enzymes were inactivated by heating at 100 °C for 3 min. The cells of *Listeria monocytogenes* CVCC 1595, *Lactobacillus plantarum* CICC 6043, and *Escherichia coli* DH5α (O.D. 600 nm≈0.7) were each resuspended in PBS (5 mmol/L, pH 6.0). All samples in which pediocin was added were incubated at 25 °C for 15 min, centrifuged at 12,000 rpm for 15 min, and tested for the percentage of bacteriocin adsorbed.

The determination of ultraviolet absorption material

Purified pediocin at a final concentration of 640 AU/mL was added to *Listeria monocytogenes* cultures at the mid-logarithmic phase of growth (O.D. 600 nm≈0.5). The *Listeria monocytogenes* CVCC 1595 suspension was added with glucose, which energizes cells, at a final concentration of 10 and 100 mmol/L. Pediocin-free cultures of *Listeria monocytogenes* were used as controls. All samples in which pediocin was added were then centrifuged, and the supernatant was examined for the presence of proteins and DNA by measuring the OD at 280 and 260 nm, respectively.

Measurement of the pH gradient (ΔpH) and the membrane potential gradient (ΔΨ)

Proton-motive force was measured by the fluorescence probe method. ΔpH was monitored by 2',7'-bis-(2-carboxyethyl)-5[and 6]-carboxyfluorescein acetoxymethyl ester (BCECF-AM) as the fluorescence probe. Approximately 1 mL of *Listeria monocytogenes* CVCC 1595 culture was centrifuged, and the cells were washed with 1 mL of HEPES buffer. The cells were resuspended in HEPES buffer and made into a 10^7CFU/mL suspension. Glucose, at a final concentration of 10 mmol/L, was added to energized cells. A 1-μL portion of the 50 mg/mL BCECF-AM was added and mixed uniformly; the mixtures were incubated at 25 °C for 10 min. Subsequently, 1 μmol/L of nigericin, 1 μmol/L of valinomycin, and 640-AU/mL of pediocin were added. Pediocin-, nigericin-, and valinomycin-free cultures of *Listeria monocytogenes* were used as controls. The fluorescence value of BCECF was immediately measured by a fluorescence photometer once per minute: 5-nm slit, an excitation wavelength of 488 nm, and an emission wavelength ranging from 500 to 650 nm, and a scanning speed of 12,000 nm/min. ΔΨ was monitored by 3,3'-dipropylthiadicarbocyanine iodide [DiSC3(5)] as the fluorescence probe. The cell suspension was added with 0.4-μmol/L DiSC3(5) and 10-mmol/L glucose, until DiSC3(5) was up to the maximum absorption. Afterwards, 100 mmol of KCI was added to balance the extracellular and intracellular K^+ concentration, and then 1 μmol/L of nigericin, 1 μmol/L of valinomycin, and 640 AU/mL of pediocin were added. The fluorescence value of the DiSC3(5) was measured immediately using an excitation wavelength of 622 nm and emission wavelength of 670 nm.

Statistical analysis of data

The data reported are the means of three repetitions. The data were analyzed by using Origin 8.0 and SPSS19.0 software for Windows 7.0 (StatSoft Inc.Tulsa, OK, USA). Evaluations were based on a significance level of $P \leq 0.05$.

Results

Purification of the pediocin

Tricine–SDS–PAGE analysis of cell-free filtrates of *Pediococcus acidilactici* culture broths confirmed a bactericidal peptide band of 4 to 5 kDa (Fig. 1). The AU of purified pediocin was determined as 640 AU/mL.

The physical and chemical properties of pediocin

Purified pediocin was stably heated from 20 °C to 100 °C for up to 1 h at ; antibacterial activity was reduced to 50 % at 121 °C (Table 1). Pediocin can be preserved for 4 weeks in −20 or 4 °C without loss of activity. The activity of pediocin can be preserved better when frozen than when refrigerated. Nevertheless, both methods are feasible in preserving pediocin activity (Table 1).

Full activity was retained even after incubation at 25 °C for 2 h at pH values ranging from 1.0 to 14.0 (at increments of 1 pH unit), and the antibacterial activity of pediocin was highest at a pH of 6.0, even in different concentrations. The overall trend of the antibacterial activity of pediocin was that the activity decreased with increasing pH. At the same pH, the antibacterial activity of high-concentration pediocin was better than that of medium-concentration pediocin, and the antibacterial activity of the medium-concentration pediocin was better than that of low-concentration pediocin (Fig. 2). A high concentration of processed pediocin changed gradually with pH, with activity at a pH of 14.0. After processing, the change of the medium-concentration pediocin was not significant between pHs 1.0 and 7.0, with a sudden drop when the pH was above 7.0 and weak activity at a pH of 14.0. The low-concentration pediocin changed gradually between pHs 1.0 and 7.0, with a sudden drop when the pH was above 7.0, activity was lost at a pH of 14.0.

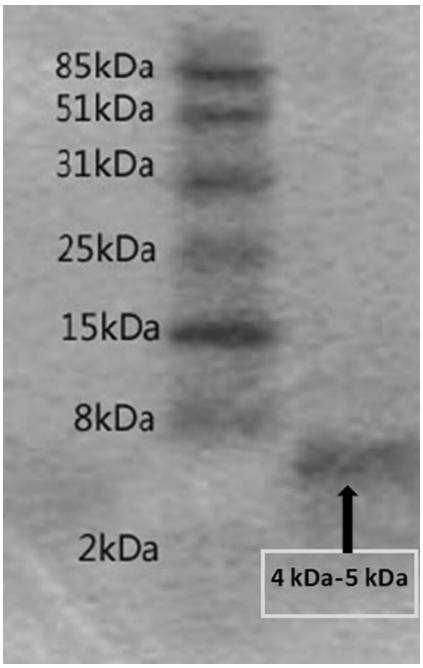

Fig. 1 Separation of pediocin by Tricine-SDS-PAGE. The arrows denote the active pediocin bands

Enzymes are main factors that influence the activity of pediocin. As a type of protein, pediocin can be easily hydrolysed by protease (Table 1). The fact that pepsin, trypsin, papain, and proteinase K are able to make pediocin lose activity confirms that pediocin is a protein.

The various biochemical properties examined in pediocin in this study were listed in Table 1, in which the properties of other described pediocins were shown for comparison.

The correlation between pediocin secondary structure and antibacterial activity

By analysing the pediocin structure in different pH treatments via CD spectroscopy, the CD spectra of different pH treatments between pH 2.0 and 10.0 were found to be quite close, and a large difference occurred at a pH of 12.0 (Fig. 3). By using DichroWeb online processing of data analysis at a pH of 12.0 (Fig. 4), this research indicated that α-helix content increased by 58 %, β-strands disappeared, and β-turns and unordered content decreased. At a pH of 6.0, α-helix content was the lowest, and β-strands and β-turns were the highest. The CD spectra of pediocin at 100 °C for 15 min and of pediocin at room temperature without any processing are identical. DichroWeb data show that the pediocin secondary structure after high-temperature treatment and after room-temperature treatment showed no differences (α-helix: 0.217 ±0.001, β-strands: 0.247±0.003, turns and unordered: 0.573 ±0.008). By using SSpro on pediocin for predicting the secondary structure (Raghava 2000), α-helix secondary structures observed were of a very low proportion, of about 6.8 %, and β-strands accounted for 25 %.

The adsorption of pediocin

Table 2 presents the influence of temperature, pH, enzymes, and different strains on the adsorption of pediocin. The adsorption of pediocin onto cells was barely influenced by temperature. The adsorption of pediocin did not differ significantly from 20 to 100 °C, as determined by statistical analysis. The adsorption of pediocin onto cells was not influenced by the treatment time, which suggests that adsorption occurred in a short period and that increasing time alone would not have an effect on sterilization. However, the adsorption of pediocin onto cells was strongly influenced by the pH of the suspending environment. Pediocin was about 98 % adsorbed between pH 6.0 and pH 6.5. At pH levels above 12.0, pediocin was not adsorbed.

Table 1 The influence of temperature, storage, pH, and proteolytic enzyme treatments on pediocin activity

Pediocin	Temperature		Storage for 4-weeks		pH	Proteolytic enzymes				Reference
	100 °C/60 min	121 °C/20 min	4 °C	−20 °C	2–10	Pepsin	Papain	Trypsin	Proteinase K	
Treatment*										
PA-1	+	±	+	+	+	−	−	−	−	This study
SA-1	+	+	+	+	+	+	+	+	+	Anastasiadou et al. 2008
PD-1	+	+	ND	ND	+	+	+	+	−	Green et al. 1997
SJ-1	+	+	ND	ND	+	ND	−	−	−	Schved et al. 1993
N5p	+	+	ND	ND	−	ND	ND	ND	ND	SträsserdeSaad et al. 1995
AcH	+	+	ND	ND	+	ND	−	−	−	Bhunia et al. 1987

[+] Activity, [±] Activity lossing above 50 %, [−] absence of any activity

ND not determined

The adsorption of pediocin onto cells treated by lysozyme and pepsin was identical to those without any processing; the adsorption of pediocin onto cells treated by trypsin, proteinase K, or papain increased by 1.25 %.

The adsorption of pediocin onto *Listeria monocytogenes* CVCC 1595 and *Lactobacillus plantarum* CICC 6043 was identical, which reached 98.44 % (Table 2). Pediocin had no inhibition action on *Escherichia coli* DH5α; neither could it be adsorbed by *E. coli* DH5α.

Cell membrane injury caused by pediocin

Cell suspensions of pediocin-treated cells were examined for the presence of DNA and protein, which gradually increased compared with the control as time increased and ultimately remained stable after 2 h (Fig. 5). However, glucose of different concentrations (no glucose, 10 mmol/L and 100 mmol/L) had no significant effect on the function of pediocin. Thus, the cells of *Listeria monocytogenes* CVCC 1595 were energized with 10 mmol/L glucose in the subsequent experiments.

ΔpH was monitored by BCECF-AM as the fluorescence probe. The fluorescence intensity of *Listeria monocytogenes* CVCC 1595 cell treated by pediocin was between that of valinomycin and nigericin, gradually increasing as time increased and ultimately remaining stable. This finding suggests that pediocin causes partial dissipation of ΔpH rather than a complete loss (Fig. 6).

However, when ΔΨ was monitored by DiSC3(5) as the fluorescence probe, the added pediocin led to a rapid rise in fluorescence intensity, resulting in damage, the same as valinomycin after 5 min. This finding suggests that pediocin leads to the complete dissipation of ΔΨ (Fig. 7).

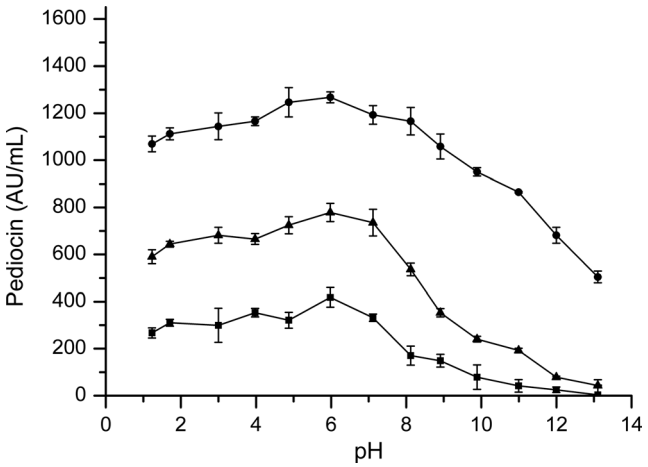

Fig. 2 Effect of pH on activity of pediocin at different concentrations. ■: pediocin of 0.03 mg/mL; ▲: pediocin of 0.1 mg/mL; ●: pediocin of 0.3 mg/mL

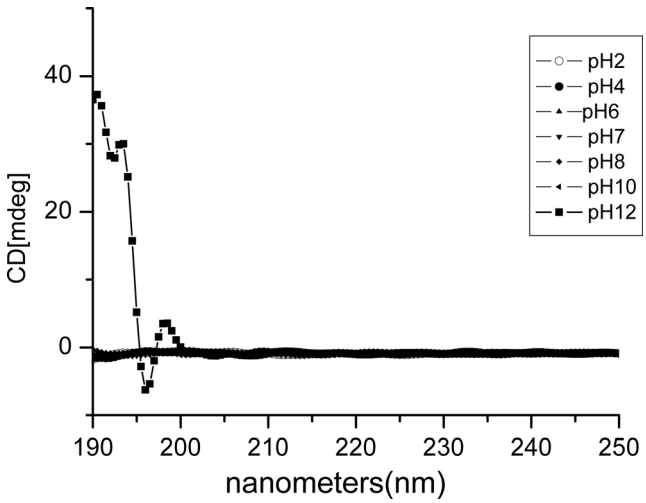

Fig. 3 Circular Dichroism spectra of pediocin at different pHs (2.0, 4.0, 6.0, 7.0, 8.0, 10.0, 12.0). ■: Circular Dichroism spectra of pediocin in pH of 12.0, characteristic peak of α-helix at 192 nm

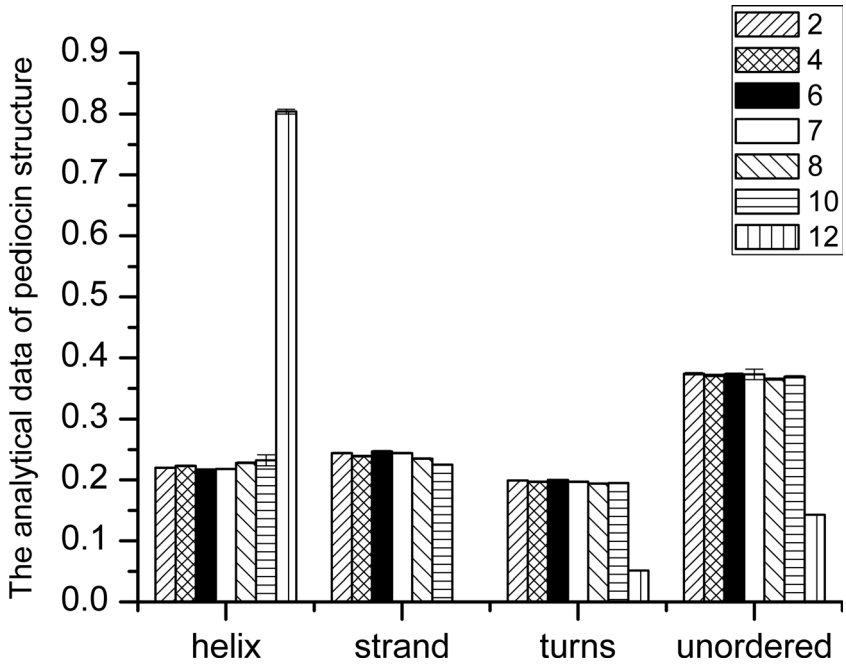

Fig. 4 Analytical data of the pediocin structure at different pHs (2.0, 4.0, 6.0, 7.0, 8.0, 10.0, 12.0)

Table 2 The influence of temperature, pH, enzymes, and different strains on the adsorption of pediocin

Adsorbed cells			Percentage of pediocin adsorbed (%)
L. monocytogenes	Temp.	−20 °C 15 min	98.44±0.49[a]
		0 °C 15 min	98.44±0.01[a]
		4 °C 15 min	98.44±0.19[a]
		25 °C 15 min	98.44±0.33[a]
		50 °C 15 min	98.44±0.06[a]
		70 °C 15 min	98.44±0.03[a]
		90 °C 15 min	98.44±0.21[a]
		100 °C 15 min	96.88±0.29[a]
	Time	1 min 25 °C	98.44±0.42[a]
		5 min 25 °C	98.44±0.15[a]
		15 min 25 °C	98.44±0.01[a]
		30 min 25 °C	98.44±0.24[a]
	pH	2.0, 25 °C 15 min	24.40±0.78[c]
		4.0, 25 °C 15 min	62.30±1.81[b]
		6.0, 25 °C 15 min	98.44±0.12[a]
		7.0, 25 °C 15 min	96.88±0.16[a]
		8.0, 25 °C 15 min	24.40±0.22[c]
		10.0, 25 °C 15 min	10.00±1.77[d]
		12.0, 25 °C 15 min	0
	Enzymes	Lysozyme, 2 mg/mL	98.44±0.09[a]
		Trypsin, 2 mg/mL	99.69±0.09[a]
		Pepsin, 2 mg/mL	98.44±0.17[a]
		Proteinase K, 2 mg/mL	99.69±0.16[a]
		Papain, 2 mg/mL	99.69±0.07[a]
	Adsorbed cells	*L. monocytogenes* CVCC 1595, 25 °C 15 min	98.44±1.25[a]
		L. plantarum CICC 6043, 25 °C 15 min	98.44±0.75[a]
		E. coli DH5α, 25 °C 15 min	0

Values in the same column with different small letters are significantly different ($p \leq 0.05$)

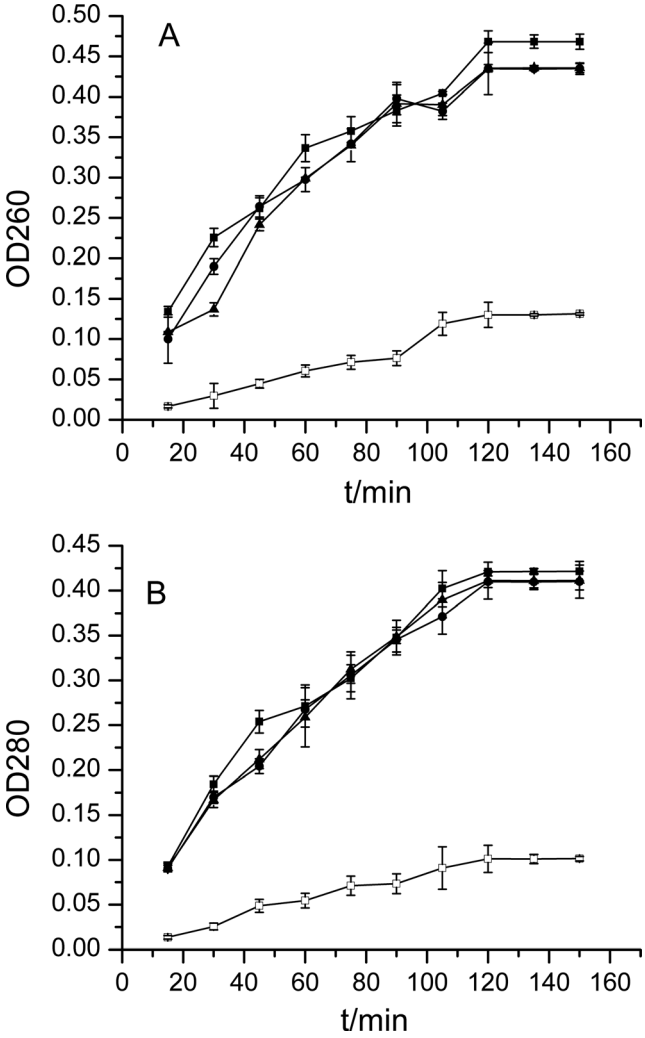

Fig. 5 Effect of pediocin on the release of intracellular nucleic acid (**a**) and proteins (**b**) from *L. monocytogenes* CVCC 1595 cells. ■: no glucose; ▲:10 mmol/L glucose; ●:100 mmol/L glucose; □: control, no pediocin, 10 mmol/L glucose

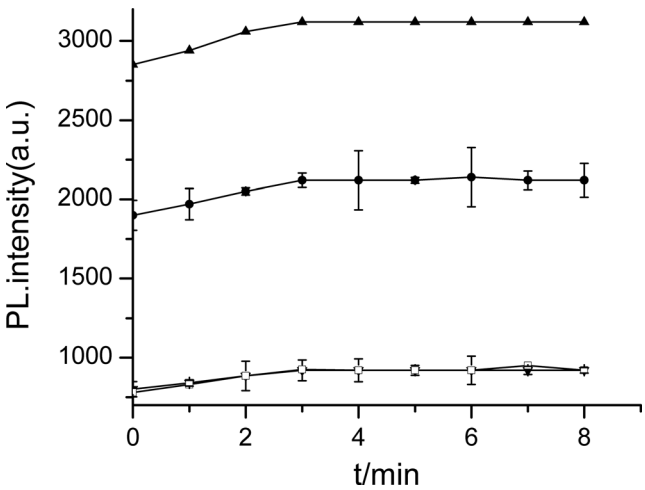

Fig. 6 The change of ΔpH of *L. monocytogenes* CVCC 1595 cells. ▲: nigericin; ▼: valinomycin; ●: pediocin added; □: control

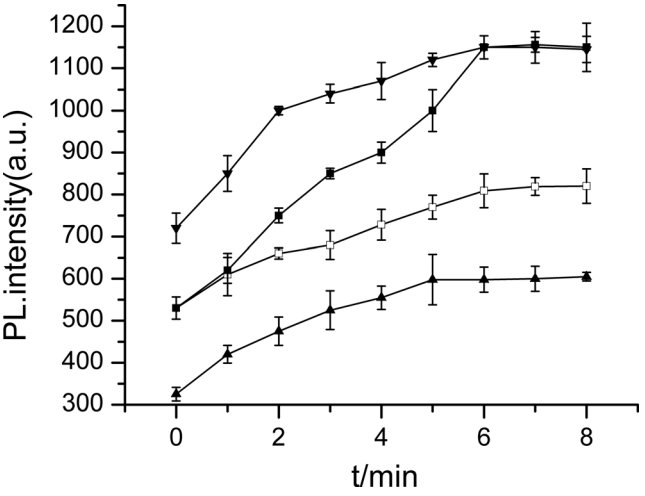

Fig. 7 The change of ΔΨ of *L. monocytogenes* CVCC 1595 cells. ▲: nigericin; ▼: valinomycin; ●: pediocin added; □: control

Discussion

The activity of pediocin associated with temperature is not obvious, probably because pediocin is a low molecular weight polypeptide that contains disulfide bridges. Klaenhammer (1993) and Nilisson et al. (2000) showed that mature pediocin contained 44 amino acid residues with low molecular weight (about 4.6 kDa) and 4 cysteine residues (cys) because it can form two disulfide bridges. In another study, Chen et al. (1997) illustrated that cysteine existing in the form of a disulfide bond was necessary for pediocin not only to maintain activity but also to provide high thermal stability. Kaur et al. (2004) studied the effects of temperature on the antimicrobial activity and structure of the C-terminal amphipathic α-helix as a receptor-binding region. Pediocin PA-1 and a chemically synthesized mutant of it, in which methionine was replaced by norleucine (for enhanced stability towards aerobic oxidation), were found to be equally active at different temperatures, whereas the peptides that lack the second disulfide bond in the C-terminal were 30 times to 50 times less antimicrobially potent at 37 °C than at 25 °C.

About 98 % of the pediocin was adsorbed at pH levels near 6.0, and the lowest adsorption (almost 0) took place at pH levels below 2.0 or above 10.0. This finding is similar to the study on adsorption conditions by Yang et al. (1992). On the basis of this property, a novel isolation method was developed for bacteriocins from four genera of lactic acid bacteria. Yang et al. made preparations of pediocin AcH, nisin, sakacin A, and leuconocin Lcm1 that were potent and concentrated by using this method. Tricine–SDS–PAGE analysis of cell-free filtrates of *Pediococcus acidilactici* culture broths confirmed a bactericidal peptide band of 4 to 5 kDa (Fig. 1). This result indicated that highly pure pediocin was obtained by using this method.

At the same pH, the antimicrobial activity of the high-concentration treatment was better than that of the low-

concentration treatment. This finding is consistent with the report of Nes et al. (1996), in which pediocin showed antibacterial activity after treatments between pH 1.0 and 9.0 at low concentrations (5 μg/mL). Antibacterial activity was observed after treatments between pH 1.0 and 12.0 by increasing the concentration (50 μg/mL), and contained antibacterial activity was observed after treatments from pH 1.0 to 13.0 by increasing the concentration further (50 μg/mL). This result showed that pediocin had acid and alkali resistance, which improved with an increasing concentration. Pediocins between pH levels 2.0 and 13.0 all had strong antibacterial activity, indicating an array of prospects in developing biological food preservatives.

When pediocin enters the digestive tract with food as a food preservative, pepsin can be broken down without side effects; this is due to many bacteriocins potentially being degraded by various types of protease. In recent years, individual organisms were shown to produce genes resistant to nisin, which encodes for nisin resistance protein (NSR). NSR is the reason for the degradation of nisin by microbial protease (Froseth and Mckay 1991). Thus, research on the bacteriocin resistance caused by protease has important practical significance. Various environmental factors, including temperature and pH, affect the defined structure and antimicrobial activity of pediocin. These characteristics are common in a number of pediocins, including pediocin SA-1 (Anastasiadou et al. 2008), pediocin PD-1 (Green et al. 1997), pediocin SJ-1 (Schved et al. 1993), and pediocin AcH (Bhunia et al. 1987).

Pediocin secondary structure was analysed using CD spectra, the results of which were identical to the data analysis of SSpro. At a pH of 12.0, α-helix content increased by 58 %, β-strands disappeared, β-turns and unordered content decreased, and pediocin activity was lost (Fig. 4). At a pH of 6.0, α-helix content was the lowest, β-strands and β-turns were the highest, and pediocin activity was the highest. At 100 °C treatment for 15 min, the disulfide bridges of pediocin cannot be destroyed. Thus, β-strand and turn structures were not destroyed, and activity remained unchanged. These results indicated that β-strands and turns in a high proportion was necessary to maintain the high activity of pediocin.

The optimum pH for adsorption of nisin (Hurst 1981) and pediocin AcH (Bhunia et al. 1991) by Gram-positive bacteria has been reported. The influence of pH on pediocin activity was consistent with that on adsorption. Different pH values led to different structures of pediocin. The β-strand and β-corner content of pediocin was highest at a pH of 6.0, possibly explained by the fact that the structure of pediocin at that pH was most suitable for adsorption. At a pH of 12.0, α-helix content increased by 58 %, β-strand disappeared, and β-turns and unordered content decreased. This decrease stopped the pediocin from being adsorbed on the surface of the bacteria. Thus, its antibacterial properties could not function. The adsorption of pediocin onto cells treated by lysozyme did not change, suggesting that the cell wall has no effect on adsorption. The adsorption of pediocin onto cells treated by proteinase K increased slightly, probably because of the effect of these types of protease on the cell membrane protein structure, either in favour of adsorption or of binding site creation. This topic requires further research.

Pediocin had no *Escherichia coli* inhibition and nor could it be adsorbed by *Escherichia coli*; this might be related to the layer of the outer membrane of the Gram-negative cells that were not conducive to pediocin adsorption. Vadyvaloo et al. (2002) showed that a large difference exists between sensitive and resistant *Listeria monocytogenes* strains in cell membrane composition. A large increase both in unsaturated phospholipids of resistant strains and in the content of short-acyl-chain fatty acids was observed, both of which might cause the increase in membrane fluidity and prevent the formation of holes on the pediocin cell membrane. Schved et al. (1994) suggested that the resistance of Gram-negative bacteria to pediocin SJ-1 is caused not only by the inability of the material to permeate the OM but also by the inability of the material to interact with the cytoplasmic membrane.

Cell suspensions of pediocin-treated cells were examined for the presence of DNA and the increase in protein levels. The results prove that the bacteriocin enables sensitive bacteria cell membranes to form holes. However, Anastasiadou et al. (2008) observed a bactericidal mode of action in studies with purified pediocin SA-1 and indicator cells of *Micrococcus luteus* at the mid-logarithmic phase of growth, in which the cell suspensions of treated cells were negative for the presence of DNA and for increased protein levels. The findings were similar to those of pediocin PD-1 and *Oenococcus oeni* cells, as reported by Green et al. (1997) and Bauer et al. (2005); they proposed that cell death is caused by membrane disruption and by a rather slow process of metabolite loss rather than by immediate loss, as is the case with nisin.

ΔpH and ΔΨ were monitored by BCECF-AM and DiSC3(5) as the fluorescence probe, in which results suggested that pediocin caused a partial dissipation of ΔpH rather than a complete loss and complete dissipation of ΔΨ. The cell deaths in the end are caused by the generation of pores in the membrane, leading to the dissipation of the PMF. These results suggested that the cytoplasmic membrane of Gram-positive bacteria is the target of pediocin, consistent with the research of Bauer et al. (2005). According to Bauer et al. (2005), the primary mode of action of pediocin PD-1 was most probably caused by pore formation, as indicated by the efflux of K^+ from metabolically active cells of *Oenococcus oeni*, which eventually led to the loss of PMF (Papagianni 2003). Given that PMF includes membrane potential (ΔΨ) and the difference in hydrogen concentration (ΔpH), experiments showed that pediocin can lead to a complete loss of ΔΨ and partial loss of ΔpH. This finding is different from that of nisin because nisin would lead to a complete loss of ΔΨ and ΔpH, according to the research of Montville and Chen (1998).

Given the loss of PMF, ATP synthesis was blocked, and cells could not absorb nutrients such as amino acids. In addition, intracellular accumulation of macromolecular materials, such as amino acids and nucleic acids, would leak out. Reports showed that original intracellular ATP would not leak, probably because of the formation of small holes. Nes and Holo (2000) reported the formation of relatively specific holes, by which PMF across the membrane might be consumed, in the bacteriocin II class. Montville and Bruno (1994) reported that a similar mechanism resulted in the consumption of proton potential power by pediocin. However, some exceptions have been observed in a variety of antibacterial systems. Specifically, two peptide bacteriocins, such as lacticin 3127 and lactococcin G, could lead to the depletion of selective membrane potential and the internal collapse of the final pH gradient of ATP hydrolysis. In addition, peptide bacteriocins, such as plantaricins EF, plantaricins JK, and acidocin J1132, could make target cells increase permeability and non-selectively consume the membrane potential and pH gradient (McAuliff et al. 1998).

This study discussed the antibacterial mechanism of pediocin, and the result showed that the antimicrobial activity might be attributed to the creation of pores in the cell membrane. These pores led to dissolution of intracellular nucleic acid and protein, dissipation of the PMF, and prevention of materials needed for energy synthesis from entering the cells, ultimately leading to cell death.

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