Purification and Characterisation of the Bacteriocin Produced by *Lactobacillus plantarum*, Isolated from Chinese Pickle

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Abstract

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119 strains of lactic acid bacteria from Chinese pickle were tested for production of antimicrobial compounds (bacteriocins). Among them, strain C8 showed strongly antibacterial activity against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739. Strain C8 was identified as *Lactobacillus plantarum* based on phenotypical, physiological tests and 16S rDNA identification. The antibacterial substance produced by strain C8 was sensitive to protease but not affected by lipase and amylase and designated as bacteriocin C8. Bacteriocin was purified by salting-out, dialysis and Sephadex G50 column chromatography. Based on SDS-PAGE, bacteriocin C8 was 16.5 kDa in size, which was different from that of other bacteriocins and it might be a novel bacteriocin. Bacteriocin C8 showed a wide range of antimicrobial activity especially as it inhibited some Gram-negative bacteria. This bacteriocin was heat resistant (20 min at 121°C) and stable in the pH range of 3 to 6.

Keywords: antibacterial activity; identification; lactic acid bacteria; resistant

The spontaneous pickle is a vegetable product which has a long history and local characteristics in China and is favourite in the Chinese population. Chinese pickle is known to be the product of mixed fermentation carried out by lactic acid bacteria (LAB), which are natural bacteria. Diverse groups of LAB have been isolated from fermented vegetables, such as *Leuconostoc, Lactobacillus, Lactococcus, Pediococcus,* etc. (Mheen & Kwon 1984). During the fermentation, LAB utilise carbohydrate substrates available in the fermentation system and produce organic acids, especially lactic acid, as a part of their metabolites. These acids not only play an important role in the taste and aroma of the product but also lower the product's pH, which is one of the key factors to ensure quality and safety. It has been reported that many LAB can produce bacteriocins which are active against closely related species (Lee *et al*. 2011).

The bacteriocins produced by LAB have been classified into four groups according to their biochemical characteristics. Because bacteriocins and their producers have potential applications as natural food preservatives, a number of bacteriocins have been identified and investigated (Chen & Hoover 2003). Generally, bacteriocins are peptides or proteins and different bacteriocins have different antimicrobial spectra. Some bacteriocins have almost no effect on Gram-negative bacteria, yeasts and moulds; only

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sakacin C2 (from *L. sakei*) showed an inhibitory effect on *Escherichia coli*, *Salmonella typhimurium*, and *Shigella flexneri* (Gao *et al*. 2010). There are not so many bacteriocins that are able to inhibit yeasts and a wide range of Gram-positive bacteria (Ko & Ahn 2000; Messi *et al*. 2001; Todorov & Dicks 2009). So, searching for LAB producing bacteriocin with a broad antimicrobial spectrum and identifying them still remain the focus of researchers' concerns. Traditional Chinese pickle is considered to be a promising source for useful LAB and various end products including bacteriocins.

The major objective of this study was to screen and identify the production of a bacteriocin from LAB from Chinese pickle, and then to study the biological characteristics of the bacteriocin.

MATERIAL AND METHODS

Isolation of lactic acid bacteria producing bacteriocins from Chinese pickle samples. Ten pickle juice samples of traditional Chinese pickle were collected from four provinces of China (4 samples from Heilongjiang, 2 from Liaoning, 3 from Hebei, 1 from Beijing). Serial decimal dilutions were prepared in saline solution (0.85% w/v NaCl in distilled water). Samples (0.1 ml, 10^{-5}–10^{-7} dilution) were spread on the surface of MRS (de Man, Rogosa, and Sharpe, Aoboxing, Beijing, China) agar with 1.0% (w/v) $CaCO_3$ and then they were incubated at 35°C for 2–3 days. Acid-producing bacterial colonies were picked from the agar plates, and then the isolates were purified on MRS plate three times. The colonies were primarily confirmed as LAB-dekete it by Gram staining and catalase tests. Only both Gram-positive and catalase-negative strains were selected and stored at –80°C in MRS broth with 20% (v/v) glycerol. The stock cultures were propagated twice in 10 ml of MRS broth at 35°C for 18 h before each experiment.

1% v/v of cultures was inoculated into 50 ml of MRS broth and incubated at 35°C for 18 hours. The medium was centrifuged at 8000 *g* for 10 min to remove the cells and then filtered through a filter membrane (pore size 0.22 μm). Filtrates were examined by the diameters of inhibition zones using an agar diffusion assay method; *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739 were used as indicator strains. 100 μl of the filtrates were placed into wells (6.00 mm in diameter) on MRS agar plates seeded with the indicator strains. Meanwhile, the pH of filtrates was adjusted to 5.0 with NaOH (5 mol/l), and catalase was added (50 U/ml/l; Sigma, St. Louis, USA), thus the effect of low pH and hydrogen peroxide was eliminated. Lactic acid solution (pH 5.0) was used as a positive control. After incubation at 35°C for 24 h, the diameters of inhibitory zones were determined.

Strain identification. First of all, the phenotypical and physiological properties of bacteria were tested. Strains from traditional Chinese pickle were determined by phenotypical and physiological tests including Gram staining, cell morphology, catalase tests, hydrolysis of arginine, carbohydrate fermentation, and growth ability in MRS medium supplemented with 6.5, 10, and 15% NaCl, respectively.

Subsequently, 16S rDNA of the microorganisms was determined. DNA extraction kit (TaKaRa Biotechnology, Dalian, China) was used to extract genomic DNA from LAB strains. The 16S rDNA fragment was amplified by PCR using a TaKaRa 16S rDNA Bacterial Identification PCR Kit (Code No. D310). The PCR primers are as follows: forward primer: 50-AGAGTTTGATCCTGGCTCAG-30, reverse primer: 50-GTGTGACGGGCGGTGTGTAC-30 (TaKaRa Biotechnology, Dalian, China). PCR conditions included denaturing at 94°C for 5 min followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, polymerising at 72°C for 1.5 min and at last polymerising at 72°C for 5 minutes. The PCR products were purified with TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (Code No. DV805A) and sequenced by Takara Bio. Homology searches of the 16S rDNA sequences were performed in the GenBank by the Blast program.

Purification of bacteriocin. One litre of MRS broth was inoculated with 1.0% (v/v) seed culture and cultivated at 35°C for 24 h under anaerobic conditions. Cells were removed by centrifugation (12 000 *g*, 15 min, 4°C). The supernatant was adjusted to pH 6.0 and then filtered through a filter membrane (pore size 0.22 μm). Bacteriocin in fermented broth was precipitated with ammonium sulphate (70% saturation, Sigma). The bacteriocin concentrates were obtained through salting-out and concentrated to one-tenth of the volume before precipitation with PEG20000.

The Sephadex G50 column (1.0 cm × 160 cm) chromatography system was used for further purification. The sample was eluted at a flow rate of 0.5 ml/min for 24 hours. The fractions were collected and detected for the bacteriocin activity using *S. aureus* ATCC 6538 as indicator. The fractions with antibacterial activity were collected and concentrated by vacuum freeze-drying.

Determination of molecular weight by SDS-PAGE. The molecular weight of bacteriocin was determined

by the Tris-Tricine SDS-PAGE with 5% stacking gel and 13% separating gel at 200 V for 4 hours. Purified bacteriocin along with low molecular weight markers (Sigma) was run together in one gel. Then the gel was stained with Coomassie brilliant blue R-250 (Fuchen, Tianjin, China) for 1 h and discoloured overnight.

Antimicrobial spectrum. Purified bacteriocin, concentrated by vacuum freeze-drying was used to determine the antimicrobial spectrum against indicator organisms (Table 3). The indicator LAB were statically incubated in MRS broth at 35°C for 24 hours. All the other indicator bacteria were cultivated overnight in nutrient broth (Aoboxing, Beijing, China) at 37°C with gentle agitation for 12 hours. *Saccharomyces cerevisiae* was grown in yeast extract peptone dextrose (YEPD, Aoboxing, Beijing, China) broth (120 r/min, 30°C, 18 h). Diameters of inhibition zones were also measured by Vernier Calliper (Gao *et al*. 2010).

Effects of temperature, pH and enzymes on the activity of bacteriocin. Purified bacteriocin was incubated in water bath at 60, 80, and 100°C for 20 min and in an autoclave at 121°C for 20 minutes. Meanwhile two other samples were stored at 4 and –20°C for 5 days. The effect of pH was tested by adjusting the pH value in a range from 3.0 to 10.0 and keeping for 12 hours. Finally, bacteriocin was treated with amylase (5 mg/ml), lipase (5 mg/ml), trypsin (5 mg/ml), papain (5 mg/ml) and pepsin (5 mg/ml; all Sigma) and incubated at 37°C for 12 h, respectively. For all the experiments here, *S. aureus* ATCC 6538 was used as indicator and controls were maintained without any treatment. Bacteriocin activity (AU/ml) was assayed as described (Barefoot & Klaenhammer 1983; Lewus & Montville 1991). All the experiments were carried out in duplicate.

RESULTS

Isolation of lactic acid bacteria producing bacteriocin. A total of 119 acid-producing bacteria were isolated and selected from the ten samples. All selected strains were primarily confirmed to belong to Gram-positive, catalase negative ones. Of these, 66 strains were rod-shaped bacteria and 53 strains were spherical-shaped bacteria. Among them, the cell-free filtrates of 6 strains with distinct antimicrobial activity against indicator strains were selected. After eliminating the effect of low pH and hydrogen peroxide, the cell-free filtrates of strains C8, C25 and Z31 still exhibited distinct inhibitory activity against both indicators (Table 1). However, strain C8 had stronger antagonism to both of the indicator strains than the other two strains; hence, strain C8 was selected for further research.

The antimicrobial activity of cell-free filtrate of strain C8 almost disappeared after hydrolysing by protease. It means that the substance produced by strain C8 was sensitive to protease and it was a protein.

Strain identification. As shown in Table 2, C8 was a catalase negative, Gram-positive, rod-shaped strain. It

Table 1. Inhibitory effects of the primarily selected strains (in mm)

Strain number	Indicator strains	
	S. aureus	*E. coli*
Control of lactic acid (pH 5.0)	–	–
P14 (original)	15.14 ± 0.51	10.52 ± 0.23
P14 (pH 5.0 + catalase)	-	-
C8 (original)	13.80 ± 0.11	19.50 ± 0.27
C8 (pH 5.0 + catalase)	11.68 ± 0.04	16.08 ± 0.78
C10 (original)	12.30 ± 0.25	9.84 ± 0.18
C10 (pH 5.0+ catalase)	–	–
C25 (original)	10.90 ± 0.05	13.84 ± 0.13
C25 (pH 5.0 + catalase)	8.24 ± 0.19	9.56 ± 0.09
W13 (original + catalase)	13.12 ± 0.11	11.50 ± 0.38
W13 (pH 5.0 + catalase)	10.92 ± 0.45	–
Z31 (original)	11.22 ± 0.15	10.84 ± 0.53
Z31 (pH 5.0 + catalase)	8.74 ± 0.22	7.36 ± 0.29

mm – the diameter of inhibition zone including that of Oxford cup (6.00 mm); original – supernatant after centrifugation and filtrate; pH 5.0 + catalase – supernatant after eliminating the effect of low pH and hydrogen peroxide

Table 2. Phenotypical and physiological characteristics of strain C8

Test	Reaction	Test	Reaction
Morphology	Rod	**Carbohydrates fermented**	
Gram reaction	+	Sorbitol	+
Catalase	–	Esculin	+
NH_3 from arginine	–	Cellobiose	+
Carbohydrates fermented		Maltose	+
Arabinose	+	Lactose	+
Ribose	+	Saccharose	+
Xylose	+	Trehalose	+
Galactose	+	Melibiose	+
Glucose	+	Melezitose	+
Fructose	+	Raffinose	+
Mannose	+	Growth ability in	
Sorbose	+	6.5% NaCl	+
Rhamnose	–	10% NaCl	+
Mannitol	+	15% NaCl	–

could grow in 6.5 and 10% but not in 15% NaCl. Moreover, it fermented a wide array of sugars. It could utilise arabinose, ribose, xylose, galactose, glucose, fructose, mannose, sorbose, and mannitol, but not rhamnose.

The 16S rDNA nucleotide sequence of strain C8 was a 1481-bp fragment. The 16S rDNA nucleotide sequence of strain C8 was of 99% similarity with that of *Lactobacillus plantarum* NRIC 1834 (AB362755.1). The GenBank access number of strain C8 is FJ378889.

Based on information from phenotypical, physiological and molecular testing the strain C8 was identified as *Lactobacillus plantarum*.

Purification of bacteriocin. As shown in Figure 1, the sample was separated into two fractions by gel chromatography. The eluents of the two peaks were collected respectively and then concentrated by vacuum freeze-drying. After testing, the fraction of the first peak showed distinct antibacterial activity. However, the fraction of the second peak had almost no antimicrobial activity. So the bacteriocin was obtained and used for SDS-PAGE.

Molecular weight determination of bacteriocin. The molecular weight was determined by relative mobility. As can be seen in Figure 2, the molecular weight of bacteriocin (designated bacteriocin C8) was approximately16.5 kDa. Compared with lane 3, lane 2 did not show any bands smaller than 14.4 kDa. Meanwhile, there were no bands of molecular weights larger than 14.4 kDa in lane 3. This indicated that the bacteriocin was produced by *Lactobacillus plantarum* C8, not by the proteins contained in MRS medium.

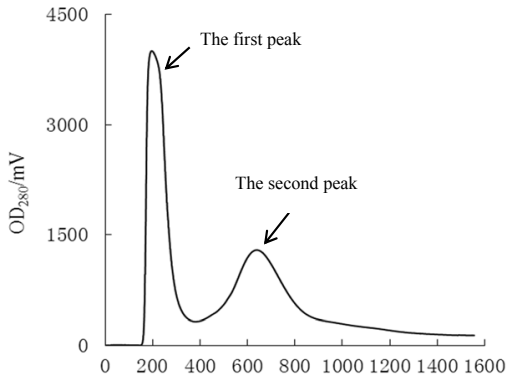
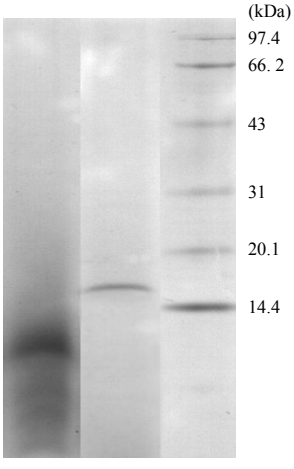


Figure 1. Gel filtration elution curve



Lane 1 – low molecular weight marker
Lane 2 – bacteriocin
Lane 3 – MRS medium

Figure 2. SDS–PAGE of bacteriocin

Table 3. Antimicrobial spectrum of bacteriocin

Indicator strains	Source[1]	Diameter inhibition zone (mm)[2]
Lactobacillus salivarius	CICC 23182	10.24 ± 0.22
Lactobacillus casei I	CICC 23185	13.47 ± 0.19
Lactobacillus plantarum	CICC 23123	9.76 ± 0.15
Lactobacillus plantarum	CICC 23165	11.96 ± 0.16
Lactobacillus rhamnosus	ATCC 7469	9.22 ± 0.19
Lactobacillus acidophilus	ATCC 4356	9.93 ± 0.31
Lactobacillus murinus	CICC 23150	–
Lactobacillus plantarum	CICC 22133	9.09 ± 0.35
Bacillus subtilis	ATCC 6633	–
Pediococcus pentosaceus	CICC 23189	14.81 ± 0.76
Lactococcus lactis	CICC 23191	8.59 ± 0.18
Enterococcus faecium	CICC 22264	12.20 ± 0.97
Enterococcus faecalis	CICC 23215	14.31 ± 0.42
Enterococcus hirae	CICC 23209	12.81 ± 0.38
Staphylococcus aureus	ATCC 6538	12.55 ± 0.33
Escherichia coli	ATCC 8739	17.28 ± 0.26
Myroides odoratus	CICC 23245	26.45 ± 2.43
Sphingobacterium thalpophilum	CICC 23242	22.72 ± 0.67
Pseudomonas fluorescens	CICC 23250	29.68 ± 1.18
Saccharomyces cerevisiae	CICC 32356	–

[1]ATCC – American type culture collection; CICC – China center of industrial culture collection; [2]diameter of inhibition zone including that of Oxford cup (6.00 mm)

Therefore, it was verified that the 16.5 kDa band was the antimicrobial peptide.

Antimicrobial spectrum. Bacteriocin C8 could significantly inhibit *Lactobacillus salivarius*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *Pediococcus pentosaceus*, *Lactococcus lacti*, *Enterococcus faecium*, *E. faecalis*, *E. hirae*, *Staphylococcus aureus*, *Escherichia coli*, *Myroides odoratus*, *Sphingobacterium thalpophilum* and *Pseudomonas fluorescens* but it did not inhibit *Bacillus subtilis*, *L. murinus*, and *Saccharomyces cerevisiae*. It exhibited a broad antimicrobial activity (Table 3).

Effects of temperature, pH and enzymes on the bacteriocin. The bacteriocin had good heat stability (Table 4). The residual activity of bacteriocin was maintained after heating, with 94.6% activity remaining even after being treated at 121°C for 20 minutes. Also, its bacteriocin activity remained stable at pH values between 3.0 and 6.0. However, the antibacterial activity disappeared entirely at pH values between 7.0 and 10.0, which indicates that the bacteriocin had no antibacterial effect under alkaline conditions. Data of

Table 4. Effects of temperature, pH and enzymes on the bacteriocin activity

Treatment		Residual bacteriocin activity (%)
Temperature (°C)	60 (20 min)	98.5
	80 (20 min)	97.3
	100 (20 min)	96.5
	121 (20 min)	94.6
	4 (5 days)	99.8
	–20 (5 days)	96.3
pH	3.0	92.6
	4.0	95.7
	5.0	100.0
	6.0	68.4
	7.0	0.0
	8.0	0.0
	9.0	0.0
	10.0	0.0
Enzymes	lipase	100.0
	amylase	100.0
	trypsin; pepsin; papain	0.0

The bacteriocin activity of the untreated sample was 260 AU/ ml, and the data of others were not shown

treatment with enzymes indicated that the antibacterial activity did not change after treating with lipase and amylase. However, proteolytic enzymes had an obvious effect and the antibacterial activity was completely disrupted. This proved that the bacteriocin produced by *Lactobacillus plantarum* C8 was a peptide.

DISCUSSION

LAB are widely distributed in nature and generally recognised as a safe group of microorganisms. Fermented vegetables are good sources of LAB. Representatives of some important genera are found in fermented vegetables such as *Leuconostoc*, *Lactobacillus*, *Lactococcus*, and *Pediococcus*. It was reported that *Leuconostoc* sp. was the main species in the early stages of fermentation, while *Lactobacillus* sp. became predominant with the pH value gradually falling to 4.0 (Mheen & Kwon 1984). Several research groups have successively isolated some LAB which can produce bacteriocin (Deraz *et al*. 2005; Todorov & Dicks 2009; Lee *et al*. 2011). In this study, 119 LAB were isolated from traditional Chinese pickle. Among them, the cells of 53 strains were globular and the rest of them were rod-shaped. The 53 strains belonged to the species *Leuconostoc mesenteroides*, *Lc. pseudome-*

senteroides, and *Pediococcus pentosus* and the other 66 strains belonged to *Lactobacillus plantarum*, and *L.pentosus* (data not shown). Among 119 LAB, strain C8 showed strong inhibition of *S. aureus* ATCC 6538 and *E. coli* ATCC 8739. This strain was identified as *L. plantarum* by phenotypical, physiological tests and 16S rDNA identification.

So far, various bacteriocins isolated from LAB in meat and dairy products have been reported. These bacteriocins are as follows: acidocin D20079 (6.6 kDa) produced by *L. acidophilus* DSM 20079 (Deraz *et al*. 2005), bacteriocin KCA2386 (8.1 kDa) produced by *Lactococcus lactis* (Ko & Ahn 2000), plantaricin 35d (4.5 kDa) produced by *L. plantarum* 35d (Messi *et al*. 2001), bacteriocin ST44AM (6.5 kDa) from *Pediococcus pentosaceus* ST44AM (Todorov & Dicks 2009), bacteriocin AMA-K (2.9kDa) from *L. plantarum* AMA-K [24], bacteriocin ST414BZ (3.7 kDa) from *L. plantarum* ST414BZ (Todorov & Dicks 2010), sakacin C2 (5.5 kDa) from *L. sakei* C2 (Gao *et al*. 2010), etc. The molecular weights generally range from 3 kDa to 10 kDa. Bacteriocins with the molecular weights higher than 10 kDa are not common. In this paper, the molecular weight of bacteriocin C8 was 16.5 kDa. This is not within the range of most bacteriocins reported before.

Bacteriocin C8 showed a wide activity spectrum. This is different from most of the bacteriocins which inhibit only closely related strains (Hata *et al*. 2010). Generally, bacteriocins were reported to exhibit a similar narrow spectrum of activity, and Gram-negative bacteria were mostly resistant to the bacteriocins of LAB. Inhibitory effects of Gram-positive strains on Gram-negative bacteria are less prevalent. For example, plantaricin ASM1 and bacteriocin KS were reported to have no effect on Gram-negative bacteria (Kawamoto *et al*. 2002; Hata *et al*. 2010). Bacteriocin ST8KF produced by *L. plantarum* ST8KF only inhibited the growth of *Enterococcus mundtii*, *L. curvatus*, *L. salivarius*, and *Listeria innocua* (Powella & Witthuhna 2007). Plantaricin 35d from *L. plantarum* 35d showed the inhibition only against related strains (Messi *et al*. 2001). Our research indicated that bacteriocin C8 has inhibitory activity against not only many Gram-positive but also Gram-negative bacteria; it has a strong inhibitory effect on some Gram-negative bacteria (*Escherichia coli*, *Myroides odoratus*, *Pseudomonas fluorescens*, and *Sphingobacterium thalpophilum*). This was not frequently seen from previous reports.

In addition, bacteriocin C8 was heat-stable. It retained 94.6% of its activity after 20 min at 121°C. This result was similar to plantaricin D (Franz *et al*. 1998), plantaricin TF711 (Hernandez *et al*. 2005), and plantaricin 423 (van Reenen *et al*. 1998). On the other hand, some plantaricins were heat sensitive, e.g. bacteriocin produced by *L. plantarum* ATCC 8014 (Bradley *et al*. 2005) and plantaricin UG1 (Enan *et al*. 1996). However, the inhibitory activity of bacteriocin C8 was in a narrow pH range; when the pH values were above 6.0, all inhibitory activity was lost. This phenomenon is common among investigators (van Reenen *et al*. 1998). There were also many bacteriocins with a wide range of pH values, such as bacteriocin J23 with a pH range of 1.0 to 12.0 (Rojo-Bezares *et al*. 2007) and plantaricin TF711 (Hernandez *et al*. 2005). As expected, bacteriocin C8 was sensitive to protease but insensitive to lipase and amylase, which indicated its peptide nature.

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