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Original Research Article

Purification, Characterization and Antibacterial Mechanism of Bacteriocin from *Lactobacillus Acidophilus* XH1

Ruixiang Zhao[1]*, Gaili Duan[1], Tianyou Yang[2], Shengyang Niu[1] and Ying Wang[1]
[1]School of Food Science, [2]College of Life Science and Technology, Henan Institute of Science and Technology, Xinxiang 453003, China

For correspondence: **Email: zhaoruixiang@hist.edu.cn*

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Abstract

Purpose: *To carry out the extraction, purification and biological characterization, and assess the antibacterial activity of bacteriocin from Lactobacillus acidophilus XH1.*
Methods: *Chloroform extraction method was used for bacteriocin extraction while characterization of bacteriocin was carried out by flat-dug well agar diffusion assay. The antibacterial mechanisms of bacteriocin were examined by scanning electron microscopy and atomic emission spectroscopy. The molecular weight of lactobacillin XH1 was measured using Tricine - SDS - PAGE electrophoresis.*
Results: *The bacteriocin (lactobacillin XH1) inhibited Escherichia coli, Staphylococcus aureus and Bacillus anthracis. It showed a wide range of antimicrobial activity at pH 1.0 - 5.0 while at 37 – 120 °C, it was sensitive to trypsin, pepsin and papain, but insensitive to proteinase K and neutral protease. The intracellular UV-absorbing substances,, namely, lactate dehydrogenase macromolecules, K^+ and ATP of E. coli, decreased rapidly. The molecular weight of lactobacillin XH1 was approximately 16 kDa.*
Conclusion: *Lactobacillin XH1 is a broad-spectrum antimicrobial substance that is thermostable. Its antibacterial mechanism on Escherichia coli is similar to that of bacteriocins on Gram-positive bacteria. The agent is a hydrophobic protein with more acidic groups.*

Keywords: *Lactobacillus acidophilus, Lactobacillin, Bacteriocin, Purification, Antibacterial mechanism, Atomic emission spectroscopy*

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INTRODUCTION

Lactobacillus acidophilus is a nonpathogenic member of the normal intestinal microflora. It has the characteristics of relieving lactose intolerance, changing the intestinal environment and improving immunity to promote physical health. Recent studies in the field of bacteriocin produced by *L. acidophilus* have demonstrated that the bacteriocin has a broad spectrum in terms of inhibition, not only on gram-positive bacteria but also on gram-negative bacterium and fungi [1]. Lactic acid bacteria bacteriocin is a kind of antibacterial polypeptide, protein or protein compound [2-4] produced by the mechanism of ribosome synthesis in the process of metabolism.

Recently, bacteriocin separation methods mainly contain organic solvents, vacuum concentration, cell adsorption and desorption, acid extraction, adsorbent adsorption, membrane separation technique and so on, but the separation results were different. Sankar *et al* [5] had isolated the bacteriocin produced by *Lactobiacillus plantarum* from milk by using the salting out method, the recovery was up to 63.1 %. This method was then proceeded with/followed by ion exchange,

gel-filtration, reversion phase chromatography and hydrophobic chromatography methods which were adopted to purify bacteriocin. However, lactic acid bacteria bacteriocin was separated mainly by adopting ammonium sulfate salting-out crude extract method, followed by gel-filtration or ion exchange to purify them, and finally, the active fractions were pooled through reversion phase chromatography.

However, few bacteriocins can be purified using the same method. The purification strategies used for each type of bacteria depend on their properties and the experimental conditions [6,7]. The antibacterial mechanism of the bacteriocin, Nisin, Sakacin and Pedioncin PA-1, from lactic acid bacteria bacteriocin had been researched deeply [8]. By forming holes in the cell walls of sensitive bacteria, intracellular substances leak out and proton motive is force dissipated [9], but there has been no study of the antibacterial mechanism of *L. acidophilus* bacteriocin in Gram-negative bacteria. The objective of the study was to produce a broad spectrum antimicrobial bacteriocin from *L. acidophilus* XH1, as well as carry out purification, characterization and elucidation of antibacterial mechanism on the agent.

EXPERIMENTAL

Bacterial strains and culture media

The bacteriocin producer-strain *Lactobacillus acidophilus* XH1 (*L. acidophilus* XH1) was obtained from the Department of Microbial Fermentation Engineering and Industry, University of Technical, Lodz, Poland, domesticated and preserved by our laboratory. In this study, *L. acidophilus* XH1 was propagated twice by 3 % inoculum in Man Rogosa Sharpe Medium (MRS) broth (Difco Laboratories, Detroit, MI, USA) for 16 - 18 h at 37 °C before experimental use. The sensitive indicator strains *Escherichia coli* JM109, *Staphylococcus aureus* ATCC6538, *Bacillus anthracis* were provided by the Microbiology Laboratory of the Department of Food Science, Henan Institute of Science and Technology.

Extraction of bacteriocin

L. acidophilus XH1 was cultivated for 16 - 18 h in 100 mL of MRS broth (pH 6.2) at 37 °C to logarithmic growth phase. Cell-free supernatants were obtained by centrifugation of cultures at 8000r/min at 4 °C for 15 min, filtered through a 0.45 μm pore size sterilized filters and stored at 4 °C until use.

Cells were removed by centrifugation. The supernatant was mixed with an equal volume of chloroform shaken vigorously for 20 min and kept overnight at 4 °C [10]. A floating interfacial precipitate was carefully collected. After centrifugation, the pellet was collected and resuspended in 1 mL of PBS (Phosphate Buffer Solution) buffer (0.05 mol/L pH 7.0). Then vacuum concentrated and repeated 3 times. It was then centrifuged at 10, 000 r/min for 15 min and the pellet was resuspended in 5 mL ultrapure water. The culture pH was adjusted to 2.0 with hydrochloric acid. The determination of the inhibitory effect of Lactobacillin XH1 on indicator bacteria was carried out with the flat-dug wells agar diffusion method [11]. Petri dish with three holes (6 mm) were prepared and filled with 80 μl cell-free supernatants. The inoculated plates were incubated for 24 h at 37 °C. The diameter of the inhibition zone was measured with calipers [12].

Effect of heat, pH and different enzymes on bacteriocin activity

To determine the heat stability, the bacteriocin crude extract was heated at 37, 80, 100 and 121 °C for 15 min and its residual antibacterial activity to *E. coli* was determined by flat-dug wells agar diffusion assay.

The sensitivity of the antibacterial activity at different pH values was estimated by adjusting the pH of supernatant sample from pH 1.0 to 7.0 with 1.0 mol/L HCl or 1.0 mol/L NaOH. After 1 h of incubation at 37 °C, the residual activity was tested as described earlier. The different pH of sterile water was used as control.

The optimum pH values of each enzyme were adjusted, 5 samples of bacteriocin crude extract as described before were treated for 2 h, 37 °C with 1 mg/mL final concentration of different enzymes viz Trypsin (pH 7.6), pepsin (2.0), Papain (5.6), neutral protease (7.0), proteinase K (8.6) (all Sigma, USA). Residual activity was determined by flat-dug wells agar diffusion assay. Untreated samples were used as control.

Bacteriocin purification

10 mL bacteriocin crude extract was filtered through 0.22 μm pore size sterilized filters and applied to cellufine A-500 and cellufine C-500 (1.6 × 30 cm) column equilibrated with 0.05 M PBS pH 7.0 at a flow rate of 1 ml/min. The peptide was eluted by a stepwise gradient-elution (0–1.0 mol/mL NaCl in 0.05 mol/mL PBS pH 7.0). Fractions of 5 mL were collected and detected at

280 nm, respectively, the anti-microbial activity was determined. The active fractions were pooled and dialyzed. Activity was determined by flat-dug wells agar diffusion assay.

Molecular weight determination by Tricine-SDS-PAGE

The method of Schagger and von Jagow [13] was used for Tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Two gels, each composed of 5 % stacking gel and 12 % separating gel were prepared. 20 µL aliquot of bacteriocin sample was mixed with 20 µL twofold concentrated sample buffer and heated for 5 min at 60 °C. Polypeptide molecular mass standards from Sigma (94.0 – 14.4 kDa) were included in each gel. Electrophoresis was run at constant voltage (30 V in the stacking gel and at 150 V during the rest of separation).

Detecting the antibacterial mechanism of bacteriocin

The logarithmic phase of mononuclear cell proliferation of E. coli, centrifugation at 8000r/min at 4 °C for 10 min, precipitation was washed in pH 7.0, 2.5 mmol/L HEPES buffer (Sigma, USA), then suspended in the buffer. To ascertain any inhibitory effects of bacteriocin against pathogenic strains bacteria (355.6 AU/mL), 2 mL Lactobacillin XH1 was added to 10 mL suspension of indicator strains of *E. coli* and 3 h incubation at 37 °C. Then the sample was fixed with 1 % (v/v) glutaraldehyde for 2 h, rinsed by PBS buffer for three times, each time for 5 min, successively with 60 %, 70 % ethanol dehydrated for 10 min, centrifuged, the precipitate was concentrated by vacuum freeze drying, gold-plated metal spraying 90 s, and then examined by electron scanning microscope (Quanta 200 scanning electron microscope of FEI Company, USA) [14].

Lactobacillin XH1 (355.6 AU/mL) was added to suspension of indicator strains of *E. coli*, 3 h incubation at 37 °C, 0.5 mL sample was taken once every half-hour and placed on ice, then centrifuged at 8000 r/min at 0 °C for 10 min. The supernatant was divided into three parts. Two parts were used to determine the UV-absorbing substances at 260 nm and the Lactate Dehydrogenase (LDH) efflux from *E. coli* cells by Multifunctional Microplate Reader (Thermo, German), and the third for determination of the K^+ efflux by Optima 2100 DV atomic emission spectroscopy (PE, USA). The bacteria cells were suspended in 1 mL, 5 % v/v trichloroacetic acid and kept overnight at -20 °C. Each suspension was incubated at 95 °C for 10 min and added to 4 mL ultrapure water. Centrifugation at 10,000 r/min at 0 °C for 15 min, the cell-free supernatant determined the concentration of intracellular K^+ from *E. coli* cells.

The method described by Sprott *et al* [15] and incorporating an ATP fluorescence detector (Promega, Beijing) was used to determine intracellular and extracellular ATP from *E. coli*.

Statistical analysis

The data are shown as the mean ± standard deviation (SD, n = 5). The results obtained were analyzed using SPSS 18.0 program for Windows (Munich, Germany) and by analysis of variance (ANOVA) with significance level set at $p = 0.05$.

RESULTS

Preliminary characteristics of crude bacteriocin

The inhibitory activity of bacteriocin, as measured by the diameter of the zone of inhibition was 15.0, 14.5, and 15.0 mm for *E. coli, S. aureus* and *B. anthracis*, respectively.

The antibacterial activity of crude bacteriocin from *L. acidophilus* subjected to varying elevated temperature was relatively unchanged for *E. coli* (Table 1). Bacteriocin showed thermostability over a wide range of temperature from 37 to 121 °C for 20 min. Bacteriocin appeared to be active over the range of pH 1.0 to 5.0, and the antibacterial activity decreased with increasing pH value. The optimum pH value was 2.0 to 3.0. The antibacterial activity of bacteriocin was sensitive to trypsin, pepsin and papain, and insensitive to proteinase K and neutral protease (Table 1).

Purification of bacteriocin

The bacteriocin was partially purified as described in Materials and Methods. For each step of the antimicrobial activity was determined as shown in Table 2. The cell free culture supernatant (80 AU/ml) was extracted with chloroform. Then the precipitate redissolved in ultra pure water was 336.84 AU/ml. Finally by cellufine A-500 column was 380.60 AU/ml. However, the eluate of cellufine C-500 column had no antimicrobial activity.

Table 1: Factors affecting the antibacterial activity (diameter of zone of inhibition) of bacteriocin against *E. coli*

Treatment	Diameter of zone of inhibition (mm) (mean±SD)	Decrease in inhibitory activity (%)
Heat treatment		
37 °C for 20min	13.75±0.03	
80 °C for 20min	13.55±0.23	
100 °C for 20min	13.50±0.09	
121 °C for 20min	13.50±0.06	
pH		
Sterile water	8.00±0.02	
1.0	15.50±0.08	
2.0	15.00±0.15	
3.0	14.00±0.14	
4.0	13.00±0.07	
5.0	11.00±0.10	
6.0	-	
7.0	-	
Enzymes		
Trypsin	10.50±0.06	30.00
Pepsin	10.05±0.11	33.30
Papain	9.50±0.11	36.70
Neutral protease	13.00±0.19	13.30
Proteinase K	12.50±0.07	16.70
Control	15.00±0.16	100.00

- = No antibacterial activity

Table 2: Purity of bacteriocin at various stages

Purification stage	Activity (AU/mL)	Volume (mL)	Recovery (%)
Cell-free culture supernatant	80.00	200.00	100.00
Chloroform extracts	336.84	10.00	21.05
Cellufine A-500 column eluate	380.60	5.00	11.89
Cellufine C-500 column eluate	-	5.00	0

- = No antibacterial activity

Molecular weight determination by Tricine - SDS-PAGE

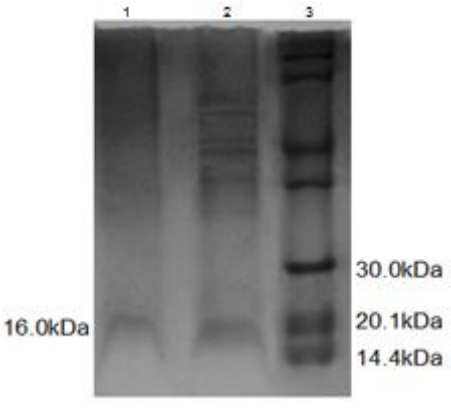

Figure 1: Tricine-SDS-PAGE of purified lactobacillin XH1 from *L. acidophilus*. (***Key:*** 1 = Cellufine a-500 column eluate; 2 = Chloroform extracts; 3 = Marker)

After ion-exchange chromatography, the bacteriocin appeared as a single band with antimicrobial activity (380.6 AU/ml) and a molecular mass of approximately 16 kDa in coomassie blue R-250 stained Tricine -SDS-PAGE gels (Figure 1).

Antibacterial mechanism

The mode of inhibition of bacteriocin was determined. Under the electron microscope, *E. coli* cells produced holes, and cell walls were partially damaged (Figure 2). The ultraviolet substances, lactate dehydrogenase macromolecules had a large leak in half an hour (Figure 3A). The concentration of intracellular K^+ of *E. coli* decreased to 0.04 μg/mL in 0.5 h (Figure 3B), and intracellular ATP of *E. coli* decreased from 112861.00 RLU to 5.89 RLU in 1h (Figure 3C).

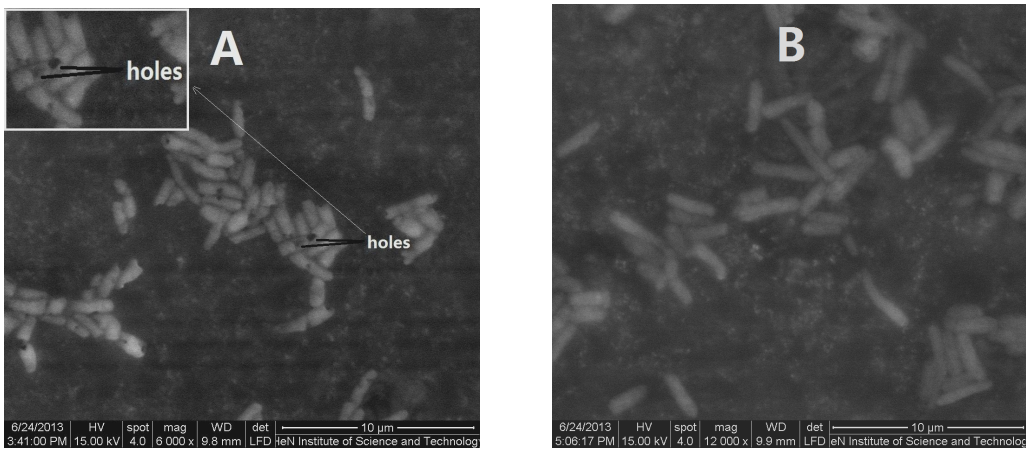

Figure 2: Effect of lactobacillin XH1 bacteriocin on the structure of *E. coli* under the scanning electron microscope. A = *E. coli* cells were treated by the bacteriocins (magnification of 6,000, scale bar = 10 μm); B = no treatment as control (magnification of 12,000, scale bar = 10 μm).

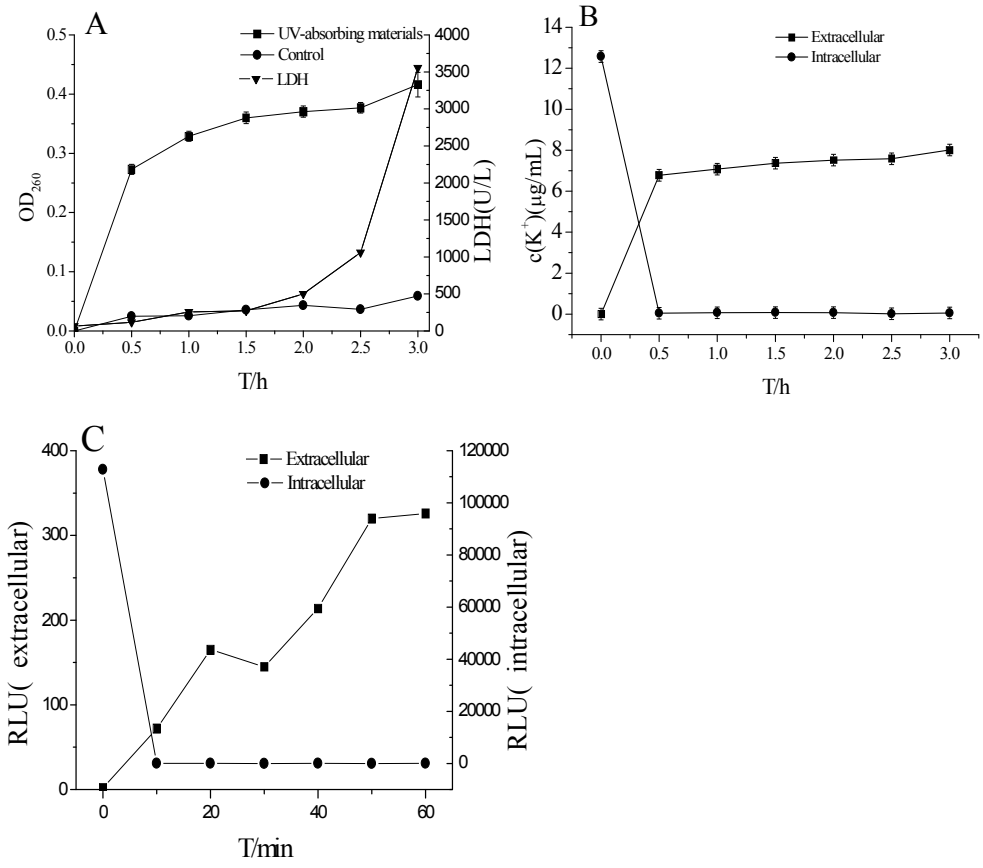

Figure 3: Effect of lactobacillin XH1 bacteriocin on the intracellular and extracellular materials in cells of *E. coli* (***Key:*** A: ■ = UV-absorbing materials from extracellular; ● = control without treatment; ▼ = LDH from extracellular; B: ■, ● = concentration of K^+ from extracellular and intracellular, respectively; C: ■, ● = ATP levels from extracellular and intracellular, respectively)

DISCUSSION

Lactobacillin XH1 inhibition of both Gram-positive and gram-negative bacteria, was indicative of broad spectrum antimicrobial substance. Similar results were reported by Maqsood *et al* [12]. The bacteriocin showed thermostability over a wide range of temperature from 37 to 121 °C for 20 min. So it can weaken the strength of sterilization and reduce the sterilization time. Similar results were found in accord with several studies [16] which directly

benefit in keeping the original nutritional value and flavor of food.

The bacteriocin appeared to be active over the range of pH 1.0 to 5.0, this indicated that Lactobacillin XH1 stability with a strong acid can be used for acidic food as biopreservative. Similar observation was illustrated by Khouiti and Simon [17]. The antibacterial activity of the bacteriocin was sensitive to trypsin, pepsin and papain, this suggested that a proteinecious nature of the bacteriocin and activity of the bacteriocin indicated was not entirely dependent on the peptide or protein fraction [18]. The eluate of cellufine A-500 column had antimicrobial activity, but cellufine C-500 column had no antimicrobial activity. It showed that the bacteriocin was a protein with more acidic groups.

Under electron microscope, *E. coli* cells produced holes, and cell walls were partially damaged. It could be due to the capacity of bacteriocin molecules embedded in the cell membrane to enhance the membrane permeability. The leakage of ultraviolet substances, lactate dehydrogenase macromolecules led to disruption of cellular matter and energy metabolism. The concentration of intracellular K^+ of *E. coli* decreased rapidly, which resulted in the imbalance of membrane potential. To maintain the membrane potential, the intracellular ATP was consumed. This could be causing the cells to die eventually. "Pore Formation" theory, described by Engelkeg *et al* [19], would be applied to Gram-negative bacteria *E. coli*. This research laid a foundation for application of lactobacillin XH1 bacteriocin and study of its structure-activity relationship.

CONCLUSION

Bacteriocin is hydrophobic in nature and negatively-charged; It is close to the uncharged or negatively-charged peptide, bacteriocin of Class I. However, bacteriocin shows thermostability over a wide range of temperature, from 37 to 121 °C for 20 min, and its molecular weight is approximately 16 kDa, which approximate to the small molecule and heat-stable peptides of Class II [20-23]. However, further studies on the chemical structure of lactobacillin XH1 are required. Its antibacterial mechanism in *Escherichia coli* is via cell membrane damage and intracellular material leakage. Thus "pore formation" theory may apply also to Lactobacillin XH1 with regard to Gram-negative bacteria.

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