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Full Length Research Paper

Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens

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The emergence of multidrug-resistant pathogens and the restriction on the use antibiotics as growth promoters in feed have drawn attention to the search for possible alternatives. Much interest has been focused on bacteriocins because they exhibit inhibitory activity against pathogens. In this study, an antibacterial substance produced by an isolated *Bacillus subtilis* strain LFB112 from Chinese herbs, was identified as bacteriocin. It was effective against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases, including *Escherichia coli, Salmonella pullorum, Pseudomonas aeruginosa, Pasteurella multocida, Clostridium perfringens, Micrococcus luteus, Streptococcus bovis* and *Staphylococcus aureus*. Two multidrug-resistant clinical isolates and a phytopathogenic yeast strain were also inhibited. The antimicrobial substance was secreted at the middle of the exponential phase, whose activity was sensitive to proteinase K and pronase E but resistant to the proteolytic action of papain, trypsin and pepsin. The antimicrobial activity was relatively heat resistant and also active over a wide range of pH 3 - 10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the active peptide had an apparent molecular weight of about 6.3 kDa. It exhibited a bactericidal activity against *S. aureus* IVDC C56005. Such characteristics indicate that this bacteriocin may be a potential candidate for alternative agents to control important pathogens in domestic animal diseases.

Key words: Domestic animal pathogen, antimicrobial activity, *Bacillus*, bacteriocin.

INTRODUCTION

Antibiotics have been utilized in the farm environment as therapeutic agents and growth promoters for over 50 years (Joerger, 2003). But the emergence of multidrug-resistant pathogens and imposed restrictions on the use of antibiotic feed additives have intensified the search for novel possible alternatives (Bedford, 2000; Wierup, 2000; Diep and Nes, 2002; Gillor et al., 2005). In this regard much interest has been focused on a group of bacteriocins due to their great potential applications in medicine (Hancock, 1997; Diep and Nes, 2002).

Bacteriocins are bacterial ribosomally synthesized antimicrobial peptides lethal to bacteria other than the producing strain (Nissen-Meyer and Nes, 1997; Joerger, 2003). Antibiotics are not included in this group because they are synthesized by multienzyme complexes and generally considered to be secondary metabolites (Jack et al., 1995). In contrast to the currently used broad-spectrum antibiotics which can kill both pathogen and commensal bacteria, some bacteriocins target specific bacterial pathogens, so they can be considered “designer drugs” (Riley and Wertz, 2002).

During the last 20 years, bacteriocins of lactic acid bacteria (LAB) have been given much attention because some of them exhibit high activity against pathogenic organisms (Diep and Nes, 2002). In contrast, bacteriocins

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from *Bacillus* have attracted little attention even though some *Bacillus spp.*, such as *Bacillus subtilis* and *Bacillus licheniformis*, are “generally recognized as safe” bacteria (Teo and Tan, 2005).

Being capable of producing a large number of antimicrobial peptides, *Bacillus* is an interesting genus to search for inhibitory substance (Bizani et al., 2005) and *B. subtilis* is one of the major producers of these substances (Foldes et al., 2000), including several bacteriocins (Sutyak et al., 2008). Unlike LAB bacteriocins, which have a narrow antimicrobial spectrum (Jack et al., 1995), bacteriocins from *Bacillus* exhibit distinct diversity in their inhibitory activities (Cordovilla et al., 1993). For example, several bacteriocins of *Bacillus* show a rather wide antimicrobial spectrum covering Gram-positive and Gram-negative bacteria (Zheng and slavik, 1999; Bizani and Brandelli, 2002; Cherif et al., 2003; Cladera-Olivera et al., 2004; Shelburne et al., 2007). Although many studies on bacteriocins from *Bacillus* have exhibited their important aspects of food safety (Hyronimus et al., 1998; Zheng and slavik, 1999; Cherif et al., 2001; Bizani and Brandelli, 2002; Martirani et al., 2002; Cherif et al., 2003; Cladera-Olivera et al., 2004; Barboza-Corona et al., 2007), few have addressed the potential application of these antimicrobial peptides to be used in animal clinical studies. Thus, there is a need for searching new inhibitory substance that has a broad spectrum of efficient antibacterial activity against domestic animals’ pathogenic bacteria.

We have screened a number of *Bacillus* strains isolated from various sources for the production of inhibitory substances against domestic animal pathogens. The selected isolate *B. subtilis* LFB112, whose bacteriocin exhibits a potential antimicrobial effect against some important domestic animal pathogens, including Gram-positive and Gram-negative bacteria. In the present study, we report the isolation, inhibitory spectrum and properties of this bacteriocin produced by *B. subtilis* LFB112 isolated strain from Chinese herbs.

MATERIALS AND METHODS

Bacteria strains and growth conditions

Five domestic animal pathogens were used as indicator bacteria in an isolation of antimicrobial-producing bacteria, including *Escherichia coli* IVDC C83901, *Escherichia coli* IVDC C83845, *Salmonella enteritidis* IVDC C79-53, *Salmonella pullorum* IVDC C79-20 and *Staphylococcus aureus* IVDC C56005. These strains were from the China Veterinary Culture Collection Center (Beijing, China). Other strains used for the antimicrobial spectrum of the inhibitory substance are reported in Table 1. The brain heart infusion (BHI) medium, blood agar (BA), deMan Rogosa Sharpe (MRS) and potato dextrose agar (PDA) were from Difco (Detroit, USA). Solid media were prepared by adding 1.5% (w/v) agar to broth media. Stock cultures were maintained at -20°C in broth supplemented with 20% (v/v) glycerol.

One of the isolated strains, designated LFB112, was studied in detail for its antimicrobial substances.

Bacterial identification

The isolated strain LFB112 was identified by API 50 CHB combined with API 20 E system (bioMerieux, France) and reconfirmed by 16S rRNA sequence analysis using ABI 3730XL DNA sequencer (Applied Biosystems, USA).

Production of bacteriocin

To study the kinetics of the bacteriocin production, the strain LFB 112 was inoculated (2.0%, v/v) into 500 mL BHI medium and incubated at 37°C in a rotary shaker at 110 cycles per min. Duplicate samples were aseptically taken at intervals over a 48-h period. One of the samples was used for measuring optical density (O.D.) at 600 nm, the other was centrifuged at 10,000 g for 15 min. Supernatant was adjusted to pH 6.8 and filtered with 0.45 µM filter membranes (Millipore, Bedford, USA). Antimicrobial activity of the supernatant was evaluated by the agar well-diffusion method (Millette et al., 2007). *S. aureus* IVDC C56005 was chosen as the indicator strain because of its high sensitivity. The minimum detectable zone measured for analytic purposes was 1 mm beyond the well diameter. One unit (U) of antimicrobial activity was defined as equal to 1 mm^2 of the zone of inhibition (Delgado et al., 2005; Barboza-Corona et al., 2007; de la Fuente-Salcido et al., 2008). Each point of activity was the average of triplicate assays.

Preparation of antimicrobial substance

The strain LFB112 (2.0% inoculum, v/v) was grown in 500 mL of BHI medium at 37°C in a rotary shake at 110 cycles per min for 16 h. Cells were removed by centrifugation at 10,000 g for 15 min. The supernatant fluid was adjusted to pH 6.8 and sterilized with 0.45 µM filter membranes and stored at 4°C until utilization. This preparation was designated as the crude bacteriocin-like substance (CBLS).

The inhibitory spectrum of antimicrobial substance

As described above, agar well-diffusion method was used to assess the antimicrobial activity of CBLS against a number of indicator strains from various collections, including Gram-positive, Gram-negative bacteria and fungi (Table 1).

Effects of enzymes, heat, pH and chemicals on antimicrobial activity

Enzymes

Samples of CBLS were treated with different enzymes (Table 2) at a final concentration of 1 mg mL^{-1} at 37°C for 2 h. Trypsin, proteinase K, pronase E and lysozyme were from Amresco (Cleveland, USA) and the other enzymes were from Sigma (St. Louis, USA). All enzymes were dissolved in buffers as recommended by the suppliers. Untreated samples plus buffer, buffer alone and enzyme solutions served as controls.

Thermal stability

To analyze thermal stability, aliquots of CBLS were exposed to different temperatures (50, 60, 70, 80, 90 and 100°C) for 15 min and to 4°C for 30 d. The resistance to lyophilization was also evaluated. Sample was lyophilized and later resuspended in the same volume of BHI medium.

Table 1. Antimicrobial activity spectrum of inhibitory substance.

Indicator strain	Source	Medium and Temperature (℃)	Activity (U)
Gram-negatives			
E. coli	IVDC C83901	BHI, 37	153.9
E. coli	IVDC C83828	BHI, 37	295.4
E. coli	IVDC C83709	BHI, 37	211.2
E. coli	IVDC C83845	BHI, 37	201.0
E. coli	multiresistant isolate	BHI, 37	124.7
H. paragallinarum	IVDC Hpg-8	BA, 37	0
P. aeruginosa	IVDC 2087	BHI, 37	213.8
P. multocida	IVDC C48-3	BA, 37	138.9
S. enteritidis	IVDC C79-53	BHI, 37	141.0
S. pullorum	multiresistant isolate	BHI, 37	136.9
S. pullorum	IVDC C79-20	BHI, 37	352.8
S. typhimurium	IVDC C77-31	BHI, 37	183.9
Gram-positives			
C. perfringens	IVDC C57-85	BA, 37	176.6
C. perfringens	IVDC C59-19	BA, 37	226.9
E. faecium	IVDC C55615	BA, 37	0
L. monocytogenes	IVDC C53005	BHI, 37	143.1
M. luteus	CMCC 28001	BHI, 37	298.7
S. aureus	IVDC 6538	BHI, 37	422.7
S. aureus	CMCC 26003	BHI, 37	265.9
S. aureus	IVDC C56005	BHI, 37	452.2
S. bovis	IVDC C55937	BA, 37	353.0
S. equi subsp. zooepidemicus	IVDC C55116	BA, 37	408.0
B. cereus	IVDC 8008	BHI, 37	0
B. licheniformis	CGMCC 1.807	BHI, 37	0
B. subtilis LFB112	isolate	BHI, 37	0
B. subtilis	CGMCC1.354	BHI, 37	0
B. subtilis	CGMCC1.769	BHI, 37	0
B. pullorum B2	isolate	MRS, 37	0
L. plantarum YJG	isolate	MRS, 37	0
Fungi			
C. utilis	CGMCC 2.120	PDA, 30	0
R. sinensis	CGMCC 2.1391	PDA, 30	143.1
A. oryzae	CGMCC 3.4382	PDA, 30	0

IVDC, China Institute of Veterinary Drug Control, Beijing, China; CMCC, China Medical Culture Collection Center, Beijing, China; CGMCC, China General Microbiological Culture Collection Center, Beijing, China; BHI, brain heart infusion; BA, columbia blood agar; MRS, deMan Rogosa Sharpe; PDA, potato dextrose agar.

pH stability

Effects of pH on antimicrobial activity were determined by adjusting the pH of the CBLS with diluted HCl and NaOH. Samples were incubated at different pH values (pH 3 - 11) for 2 h at 25℃, then neutralized to pH 6.8 and tested for antimicrobial activity.

Effect of chemicals

Different chemicals were added to the CBLS and incubated at 37℃ for 1 h before being tested for antimicrobial activity. Organic solvents (methanol, ethanol, butanol, isoamylol, chloroform, N-hexane and acetone) were used at the working concentration of 50% (v/v). The detergents Tween 20, Tween 80 and sodium dodecyl sulfate (SDS) were used at a final concentration of 1% (v/v), sodium deoxycholate was used at 1 mg mL^{-1} and Triton X-100 was used at 0.02% (v/v). DL-Dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA) and trichloroacetic acid (TCA) were used at 10 mM L^{-1}, 10 mM L^{-1} and 100 mg mL^{-1}, respectively. All chemicals were from Amresco (Cleve-land, UAS). After treatment with trichloroacetic acid (TCA), samples were centrifuged at 10,000 g for 5 min and the

Table 2. Effect of enzymes, heat and pH on antimicrobial activity.

Treatment	Residual activity (%)*
Enzymes	
Pronase E	0
Proteinase K	45
Catalase	100
Pepsin	91.3
Trypsin	85
Papain	100
Lysozyme	100
α-amylase	100
Lipase	88.5
Heat	
50°C/15 min	100
60°C /15 min	100
70°C /15 min	93
80°C /15 min	78.6
90°C /15 min	73
100°C /15 min	64.7
100°C /30 min	0
4°C /30 d	75
Freeze-dried	100
pH	
3	79
4	85.2
5	100
6	100
8	100
9	58.4
10	45
11	0

*Residual activity compared with antimicrobial activity before the treatment.
Results are means of three individual assays with an SD less than 5% about the mean.

supernatant was adjusted to pH 6.8 before testing for antimicrobial activity. Chemicals and the CBLS diluted with 8.75 g L^{-1} NaCl were used as controls.

After each treatment the samples were tested for residual activity using agar well-diffusion method against *S. aureus* IVDC C56005. All experiments were done in triplicate.

The bactericidal action on *S. aureus*

The CBLS (final activity about 180 U) was added to early exponenttial growth phase cells of *S. aureus* IVDC C56005 in 15 mL BHI medium. Sterile BHI medium was added to control tubes. The culture was incubated at 37°C, at regular interval, samples were taken to determine the O.D. at 600 nm and the number of colony-forming units (CFU) on BHI plates by the standard plate-counting methods. All assays were done in triplicate.

The precipitation of bacteriocin with ammonium sulfate

The spent culture supernatant was precipitated with ammonium sulfate at 80% saturation under chilled conditions for 18 - 24 h. The precipitated proteins were collected by centrifugation (10,000 g, 30 min), pellet was suspended in one hundredth of original culture volume of 20 mM phosphate buffer solution (PBS, pH 6.8) and dialyzed using 1 kDa cut-off membrane (Sigma) against the same buffer at 4°C for 36 h. The dialyzed proteins were stored at -20°C.

Direct detection of antimicrobial activity on Tricine-SDS-PAGE gels

Duplicate samples (10 µL) of the dialyzed proteins and low molecular mass standards (Hou-Bio, Hong Kong, China) ranging from 2 to 71 KDa were subjected to Tricine-sodium dodecylsulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). The procedure was carried out on 16% polyacrylamide gels using tricine as trailing ion (Schagger, 2006). Following electrophoresis conducted at 100 V for 90 min, the gel was cut vertically. The first part, containing the sample and protein standards, was stained with staining solution (0.25% Coomassie brilliant blue R-250, 25% isopropanol, 8% acetic acid) to determine the molecular weights of separated protein bands (Figure 3a). The other part of the gel was assayed for direct detection of inhibitory activity according to the previous method (Barboza-Corona et al., 2007) with modification. Briefly, this part was fixed (25% isopropanol, 10% acetic acid) for 60 min, washed with double-distilled water for 1 h. The gel was aseptically placed in a sterile Petri dish with 5 mL soft BHI medium (0.75% agar, w/v) containing approximately 0.1 mL overnight culture of *S. aureus* IVDC C56005. The Petri dish was incubated at 37°C for 24 h and observed for the presence of an inhibition zone.

RESULTS

Characterization of producing strain

The antimicrobial-producing strain LFB112 was isolated from Chinese herbs. It was Gram-positive, aerobic, catalase positive, rod-shaped and spore-forming bacterium. The identification of this isolate to species level according to the profiles of API tests was carried out by means of interpretation software (V3.0). The results showed that isolate LFB112 was closely related to *B. subtilis* with an identification of 96.7% and a T-value of 0.83. The complete 16S rDNA sequence (1427-bp) of the stain LFB112 was obtained. Similarity searches with sequences in the GenBank database revealed 99.2% identity with the *B. subtilis* 16S rDNA sequence (GenBank accession number DQ523502, Nielsen et al., 2007). This sequence has been submitted to GenBank and has accession number FJ527490. These results show that LFB112 is a *B. subtilis* strain.

The inhibitory activity of *B. subtilis* LFB112 was detected in both liquid and solid cultures. By using diluted samples of the cell-free supernatant, a progressive decrease in the diameter of the inhibition zone of the indicator strain was observed, without the appearance of

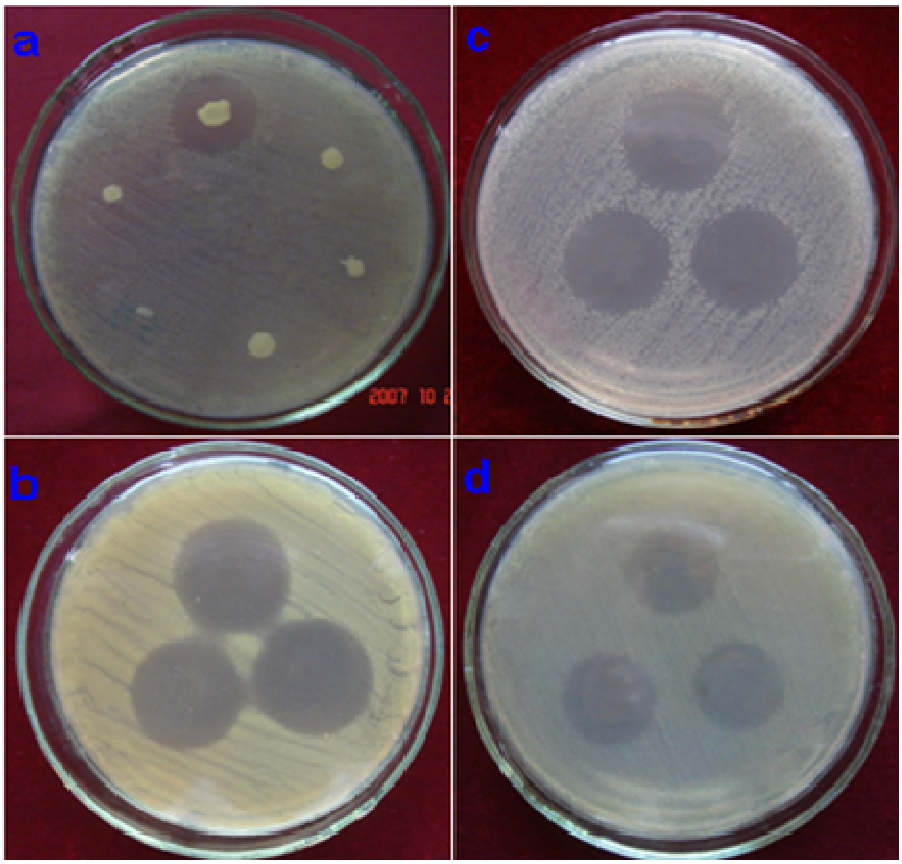

Figure 1. The antimicrobial activity of *B. subtilis* LFB112 against animal pathogens: (a) spot agar assay demonstrating inhibitory activity of *B. subtilis* LFB112 against *S. pullorum* IVDC C79-20; (b), (c), and (d) agar well-diffusion assay demonstrating inhibitory activity of bacteriocin-like substance against *S. aureus* IVDC C56005, *S. pullorum* IVDC C79-20, and *E. coli* IVDC C83845, respectively.

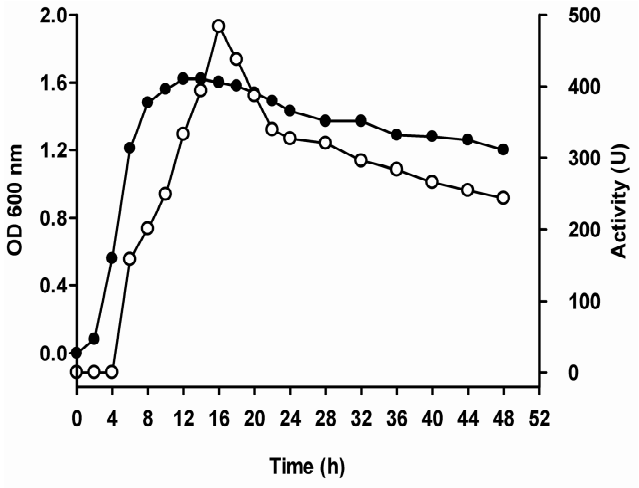

Figure 2. Kinetics of bacteriocin production during the growth of *B. subtilis* LFB112 in BHI broth at 37°C for 48 h. Optical density (O.D.) of LFB112 (black circles) and antimicrobial activity (open circles). Each point represents the mean of three independent experiments with an SD less than 5% about the mean.

plaques, which would indicate the inhibition was not caused by replication of bacteriophage. An example of the well-diffusion and spot agar assay (Paik et al., 1997) exhibiting the antibacterial activity of *B. subtilis* LFB112 against indicator strains is shown in Figure 1.

Production of bacteriocin

In order to study the production of antimicrobial compound during *B. subtilis* LFB112 growth, the inhibitory activity present in cell-free samples taken at different time intervals was measured (Figure 2). Antibacterial activity could be detected at the mid-log growth phase and quickly reached a maximum at the early stationary phase. After that, the antagonistic activity declined. This decrease may be attributed to adsorption on producer cells or to degradation by specific or non-specific proteases (Parente and Ricciardi, 1999). The kinetics of this bacteriocin was similar to those described for bacteriocins from *Bacillus thuringiensis subsp tochigiensis* (Paik et al., 1997), *B. licheniformis* P40 (Cladera-Olivera

et al., 2004) and *B. licheniformis* ZJU12 (He et al., 2006), but differed from those for Lichenin produced by *B. licheniformis* 26 L-10/3RA (Martirani et al., 2002), whose activity appeared at early stationary stage and slowly reached a maximum titer within the next 42 h. As this inhibitory substance was produced only during the exponential growth phase it can be considered a primary metabolite, like bacteriocins produced by *Enterococcus faecium* (Parente et al., 1994), *Lactobacillus amylovorus* (De Vuyst et al., 1996) and *B. licheniformis* strain (Cladera-Olivera et al., 2004).

Inhibitory spectrum

The inhibitory spectrum of bacteriocin was determined by agar well-diffusion method (Millette et al., 2007) against different indicator strains (Table 1). Inhibitory activity was observed against most pathogenic strains, including some important animal pathogens and two multidrug-resistant clinical isolates of *Escherichia coli* and *Salmonella pullorum*, as well as against a yeast strain. No activity was detected against *Lactobacillus*, *Bifidobacterium*, *Bacillus subtilis* and *B. licheniformis*, which were generally recognized as beneficial bacteria or safe bacteria. The producer strain *B. subtilis* LFB112 was not inhibited by its own bacteriocin. The inhibitory spectrum was similar to those shown by cerein 8A (Bizani and Brandelli, 2002), entomocin 9 (Cherif et al., 2003) and bacteriocin-like substance produced by *B. licheniformis* P40 (Cladera-Olivera et al., 2004), but wide compared with those of coagulin (Hyronimus et al., 1998), cerein 7 (Oscariz et al., 1999) and thuricin 7 (Cherif et al., 2001), which only inhibited Gram-positive indicator strains.

Effects of enzymes, heat, pH and chemicals on antimicrobial activity

To determine the biochemical and biophysical properties of the inhibitory substance, samples were tested for sensitivity to enzymes, chemicals, temperatures and pH values. These results are summarized in Table 2. When samples were treated with pepsin, lipase, trypsin, proteinase K and pronase E, the inhibitory activities decreased (8.7, 11.5, 15, 55 and 100%, respectively). However, little or no change in the size of the inhibitory zone was observed when the same samples were treated with papain, catalase, lysozyme and α-amylase.

The antimicrobial activity was relatively heat resistant and also active over a wide range of pH values, as full activity was retained at pH 5 - 8 and activity was more stable in acidic than basic conditions. These results were similar to coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* (Hyronimus et al. 1998). There was no loss of inhibitory activity when stored for up to 7 days at refrigerated temperature and activity was reduced to 75% at 30 days of storage. Lyophilization and resuspension of the sample did not affect the antimicrobial activity, indicating that this bacteriocin is attractive for the potential biotechnological application.

When samples were treated with SDS and TCA, no inhibitory activity was observed. After treatment with sodium deoxycholate, EDTA, chloroform and DTT, residual activity decreased significantly (19.5, 27.2, 45 and 52%, respectively). However, all other chemicals used caused no effect on antimicrobial activity.

SDS-PAGE analysis and mode of action of inhibitory substance

Direct detection of the antimicrobial activity was performed by Tricine-SDS-PAGE. As shown in the stained bands of the gel, several proteins were detected in the samples (Figure 3a). The other part overlaid with indicator strain revealed a single protein band with inhibitory activity (Figure 3b). The band had an apparent molecular mass of about 6.3 kDa, as estimated by calculating the relative migration values of standard proteins.

The effect of inhibitory activity on the growth of *S. aureus* IVDC C56005 is shown in Figure 4. The addition of CBLS dilution to about 10^6 CFU mL^{-1} of indicator strain resulted in a rapid decrease in the number of viable counts. We observed about a 500-fold reduction after 5 h incubation at 37°C. The O.D. of the treated culture with CBLS did not change significantly during the experiment (Figure 4). Other bacteriocins were also reported to exhibit such mode of action (Bhugaloo-Vial et al., 1996; Paik et al., 1997).

Based on the characteristics of the inhibitory substance produced by *B. subtilis* LFB112 and according to the criteria as previously described (Jack et al., 1995), it was characterized as a bacteriocin-like substance.

DISCUSSION

The aims of this study were to isolate antimicrobial-producing *Bacillus* spp. from various sources and to screen their bacterocins as potential natural antibacterial agents for use against animal pathogens. *B. subtilis* is a known producer of many antibiotic and antimicrobial compounds, including the bacteriocin sublancin 168 (Paik et al., 1998), bacillocin 22 (Zheng and Slavik, 1999) and subtilosin A (Shelburne et al., 2007).

Unlike some bacteriocins from *Bacillus* spp. exhibit a relatively narrow inhibitory spectrum (Naclerio et al., 1993; Paik et al., 1997; Martirani et al., 2002; Ahern et al., 2003), this bacteriocin presents a wide activity spectrum. The inhibitory activity to inhibit the growth of *E. coli* IVDC C83901, *E. coli* IVDC C83709, *S. enteritidis* IVDC C79-53, *Salmonella typhimurium* IVDC C77-31 and *Clostridium perfringens* IVDC C57-85, which are known

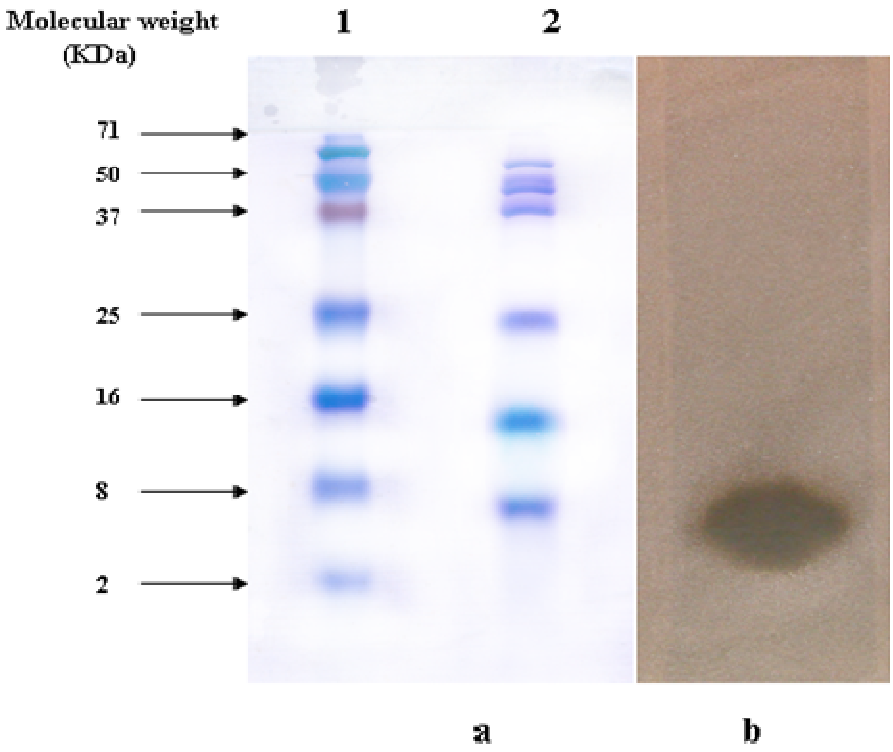

Figure 3. Tricine-SDS-PAGE analysis and direct detection of antimicrobial activity. (a) Coomassie blue-stained gel. Lane 1: molecular weight marker; lane 2: partially purified bacteriocin; (b) the inhibition zone was shown by overlaying the gel with soft BHI medium (0.75% agar) containing the *S. aureus* IVDC C56005 indicator strain.

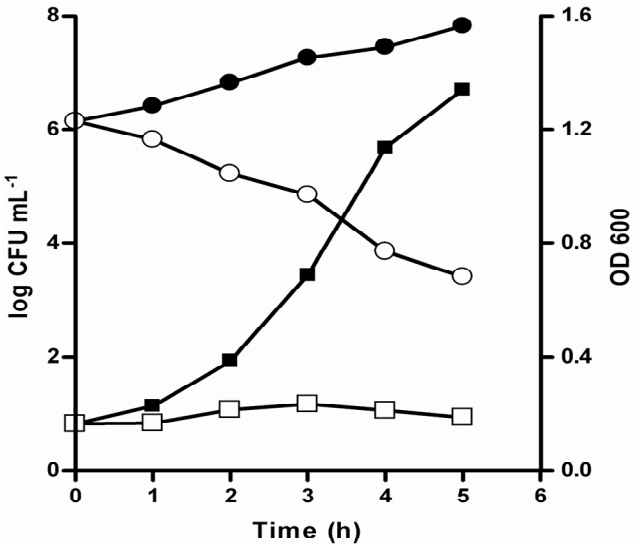

Figure 4. Effect of bacteriocin on growth of *S. aureus*. Optical density (squares) and CFU mL^{-1} (circles) were measured in control (closed) and treated cells (open). Each point represents the mean of three independent experiments with an SD less than 5% about the mean.

as the main causative agents of diarrhea in piglets (Asai et al., 2002; Fairbrother et al., 2005) and necrotic enteritis in chickens (Mcdevitt et al., 2006), is an important property to animal health. The inhibition of *Streptococcus equi subsp. zooepidemicus* and *S. aureus* involved in mastitis would be very beneficial to the pig and cattle industries. The bacteriocin was also able to inhibit the growth of *Pasteurella multocida*, which was the primary causative agent of avian cholera (Blehert et al., 2008). In addition, two multidrug-resistant clinical isolates involved in animal infections and a phytopathogenic yeast strain were also inhibited. Interestingly, the inhibitory substance was not able to inhibit the growth of *Lactobacillus plantarum* and *B. pullorum*, which constituted a major part of the normal intestinal flora in animals and humans and have been used as probiotic strains (de Vrese and Schrezenmeir, 2008), suggesting that this bacteriocin can offer some advantages over existing antibiotics which kill both pathogenic and beneficial micro-organisms. *B. subtilis* are being used as probiotics and competitive exclusion (CE) agents for both human and animal consumption, which can synthesize antimicrobial substances (e.g. bacteriocins) to prevent the colonization the gastrointestinal tract by pathogenic bacteria (Casula and Cutting, 2002). In this case, it is very important in the view of the fact that this bacteriocin had no inhibition against other *B. subtilis* strains.

Considering the bacteriocin properties, its antimicrobial activity was sensitive to microbial proteases and was lost

with TCA treatment, suggesting that it is proteinaceous (Bizani and Brandelli, 2002). Nevertheless, the fact that the inhibitory substance retained most of its activity after exposure to pepsin and trypsin, indicating that this bacteriocin may be cyclic peptides containing unusual amino acids, therefore more resistant to proteases hydrolysis (Bizani and Brandelli, 2002). These results found in the study suggest that this antimicrobial peptide possibly can survive at the intestinal environments and could be administered with feed. The inhibitory substance was partially sensitive to trypsin and chloroform, providing indirect evidence that the molecule may contain lipid (Barefoot and Klaenhammer, 1984; Teo and Tan, 2005). It can also be inferred that the antimicrobial activity could not be due to the production of organic acid or hydrogen peroxide. When the compound was treated with the reducing agent DTT, a significant decrease of inhibitory activity was observed, indicating that, at least, one disulphide bond is essential for its antibacterial activity (Eijsink et al., 1998; Miller et al., 1998; Oscariz and Pisabarro, 2000; Cherif et al., 2003). In addition, a loss of antimicrobial activity was observed when the bacteriocin was treated with the denaturant SDS, while an inhibition zone was observed clearly in the direct detection of antimicrobial activity on Tricine-SDS-PAGE gel (Figure 3b), suggesting that the protein can fold spontaneously in its correct structure easily.

The mode of action was studied using *S. aureus* as the target bacteria. A rapid decline in the number of viable counts induced by the bacteriocin was observed, while the optical density (O.D.) of the treated culture with the bacteriocin did not changed significantly during the experiments. The results suggest that the inhibitory substance acted bacteriocidally rather than bacteriostatically on the sensitive cells.

Bacteriocins have already been proposed as a promising alternative of the existing antibiotics for treatment because it is inexpensive, effective and non-toxic to animals and humans (Lewus et al., 1991; Milles et al., 1992; Cleveland et al., 2001). Some studies have evaluated the therapeutic efficacy of bacteriocins in the treatment of animal disease (Ogunbanwo et al. 2004; Cole et al. 2006). In summary, we have demonstrated the characterization of a bacteriocin produced by a *Bacillus subtilis* LFB112 strain isolated from Chinese herbs, which has large inhibitory spectrum covering various domestic animal pathogens without interference with the growth of beneficial bacteria. It makes this bacteriocin a potential candidate as antimicrobial agents and growth promoters in the farm environment. Nevertheless, more work needs to be done to arrive at such conclusion.

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