Bovicin HJ50, a novel lantibiotic produced by *Streptococcus bovis* HJ50

Haijie Xiao,1 Xiuzhu Chen,1 Meiling Chen,1 Sha Tang,3 Xin Zhao1 and Liandong Huan1,2

A bacteriocin-producing strain was isolated from raw milk and named *Streptococcus bovis* HJ50. Like most bacteriocins produced by lactic acid bacteria, bovicin HJ50 showed a narrow range of inhibiting activity. It was sensitive to trypsin, subtilisin and proteinase K. Bovicin HJ50 was extracted by n-propanol and purified by SP Sepharose Fast Flow, followed by Phenyl Superose and Sephadex G-50. Treatment of *Micrococcus flavus* NCIB8166 with boovicin HJ50 revealed potassium efflux from inside the cell in a concentration-dependent manner. The molecular mass of bovicin HJ50 was determined to be 3428 ± 3 Da. MS analysis of DTT-treated bovicin HJ50 suggested that bovicin HJ50 contains a disulfide bridge. The structural gene of bovicin HJ50 was cloned by nested PCR based on its N-terminal amino acid sequence. Sequence analysis showed that it encodes a 58 aa prepeptide consisting of an N-terminal leader sequence of 25 aa and a C-terminal propeptide domain of 33 aa. Bovicin HJ50 shows similarity to type AII lantibiotics. Chemical modification using an ethanethiol-containing reaction mixture showed that two Thr residues are modified.

INTRODUCTION

Bacteriocins are antimicrobial peptides, proteins or complex proteins produced by certain bacteria (Allison & Klaenhammer, 1999; Jack et al., 1995; McAuliffe et al., 2001). Many species of bacteria produce bacteriocins, but bacteriocins produced by lactic acid bacteria (LAB) are of particular interest because of the relationship between LAB and humans. Since nisin was found in 1928, over 70 kinds of bacteriocin produced by LAB have been found. Nisin, the best studied bacteriocin, has been approved as a food preservative in over 50 countries. During the last few decades a lot of research into bacteriocins produced by LAB has been carried out. Bacteriocins are one of the antimicrobial substances produced by LAB and it is thought that they play an important role in inhibiting other bacteria in the same environment.

Bacteriocins have been categorized into four groups according to their chemical properties (Klaenhammer, 1993): group I, lantibiotics which contain unusual amino acid residues, such as lanthionine, 3-methylanthionine, 2,3-didehydroalanine and 2,3-didehydrobutyryline; group II, small, heat-stable peptides; group III, large, heat-labile proteins; group IV, complex proteins, composed of protein plus lipid or carbohydrate. Group IV bacteriocins are somewhat questionable because of inadequate data. Bacteriocins from groups I and II are the best studied. A great deal of research into bacteriocins and their uses has been carried out, including characterization of new bacteriocins, modification of bacteriocins by protein engineering (Chen et al., 1998; Rollema et al., 1995), construction of food-grade vectors (Takala & Saris, 2002), regulation and expression of heterologous proteins (de Ruyter et al., 1996), control of flavour and other characteristics of fermented food, and pharmaceutical and veterinary applications of bacteriocin producing bacteria, etc. Originally, the potential use of bacteriocins as food preservatives stimulated research into bacteriocins produced by LAB which has led to a greater understanding of these important bacteria.

Many bacteriocin-producing LAB have been isolated from raw milk; *Lactococcus* spp., *Lactobacillus* spp. and *Leuconostoc* spp. are the most abundant. Production strains for nisin, lactcin 481 and garviecin L1-5 (Villani et al., 2001) were isolated from milk. From raw milk provided by a dairy, we isolated a strain producing a novel bacteriocin. In this paper, the biochemical and genetic characterization of this bacteriocin is studied.
METHODS

Bacterial strains and media. The bacteriocin-producing strain (Streptococcus bovis HJ50) was isolated from raw milk provided by the Qutou Dairy of Beijing and was grown anaerobically in M17 medium with 5 g glucose l⁻¹ at 37 °C. An indicator strain, Micrococcus flavus NCIB8166, was grown in S1 medium at 30 °C.

Characterization of the bacteriocin-producing strain. S. bovis HJ50 was tested for growth temperature, growth at pH 9-6, hydrolysis of arginine and asparagine, production of catalase and amylase, Voges-Proskauer reaction, growth in 6-5 % NaCl and acid production from several carbohydrates.

Total DNA was extracted according to Lewington et al. (1987). Two primers were used for 16S rDNA analysis: 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1541r (5'-AAGGAGGTGATCCAGCC-3'). PCR was performed under the following conditions: 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and polymerization at 72 °C for 3 min. The PCR product was ligated into pGEM-T vector (Promega) for DNA sequencing.

Detection of bacteriocin activity and susceptibility to proteases. A culture of S. bovis HJ50 was centrifuged to remove the cells. The cell-free supernatant was adjusted to pH 7-0 for the detection of bacteriocin activity by the well-diffusion method. A neutral solution was also used to measure activity against other bacteria to determine the antimicrobial spectrum of bovicin HJ50. Susceptibility to proteases was examined according to Aktypis et al. (1998). A neutral solution of bovicin HJ50 was also treated with trypsin, subtilisin and proteinase K at 37 °C for 1 h.

Extraction and purification of bovicin HJ50. Bovicin HJ50 was extracted with n-propanol according to Cheeseman & Berridge (1957). Briefly, a culture of S. bovis HJ50 was adjusted to pH 2 with HCL. After centrifugation, 0-1 vols n-propanol was added into the broth supernatant, then 300 g NaCl was added. The supernatant/n-propanol solution was collected and the residual broth was extracted with 30 ml n-propanol per litre of broth for a second time. All of the n-propanol solution was collected and 2 vols cold acetone was added to obtain a precipitate of bovicin HJ50. The precipitate was dissolved in 0-05 M citric acid buffer (pH 4-2) to obtain a crude extract of bovicin HJ50.

The crude extract was dialysed against 0-05 M citric acid buffer (pH 4-2) and applied to an SP Sepharose Fast Flow column previously equilibrated with the same buffer. Bovicin HJ50 was eluted with a 0-1 M NaCl gradient by using a fast performance liquid chromatography (FPLC) system. Active fractions were combined and dialysed against 0-05 M citric acid buffer (pH 4-2) containing 1-5 M NaCl. A Phenyl Superose column was used for hydrophobic interaction chromatography, eluted with a 1-5-0 M NaCl gradient. Active fractions were combined and lyophilized. Then the bacteriocin sample was applied to a Sephadex G-50 column for gel filtration. The active fractions were collected and lyophilized. Protein concentration was measured by the method of Bradford (1976).

Determination of potassium efflux. Potassium efflux was determined according to Chen & Montville (1995). Cells of M. flavus NCIB8166 were grown in S1 medium supplemented with 2-5 mmol KCl l⁻¹ at 30 °C. Cells were harvested at mid-exponential phase (OD600=0-6-0-7) for cell dry weight and potassium efflux determination. Cells were washed in 0-1 M MES buffer (pH 6-3) containing 0-2 % glucose and 0-6 mmol KCl l⁻¹ and resuspended in the same volume of MES buffer. Purified bovicin HJ50 was added to the cell suspension at different concentrations. A cell suspension with no bacteriocin added served as a blank control. One hour after treatment with bovicin HJ50, the suspension was centrifuged at 12 000 r.p.m. for 10 min to remove the cells. The supernatant was applied to a plasma spectrum (Prodigy; Leeman Labs) for determination of potassium.

MS analysis of bovicin HJ50. The molecular mass of purified bovicin HJ50 was determined by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS on a BIFLEX III TOF-MS instrument. Bovicin HJ50 treated with 4 mmol DTT l⁻¹ for 15 min at 60 °C was also used for MS analysis.

N-terminal sequence analysis of bovicin HJ50. The N-terminal sequence of purified bovicin HJ50 was determined on an Applied Biosystems 477A automatic sequence analyser by using the Edman degradation method.

Cloning of the gene encoding bovicin HJ50. Three degenerate primers: P1, P2 and P3 (Table 1), designed according to the N-terminal sequence of bovicin HJ50, were used to clone the gene encoding bovicin HJ50. Cys was tentatively used to substitute the third position because of the homology between bovicin HJ50 and type AII lantibiotics. PCR was performed with 2 μM each primer mix, P1 or P2 with P3, under the following conditions: 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 50 s, annealing at 49 °C for 50 s and polymerization at 72 °C for 20 s. A 44 bp PCR product was obtained for sequence analysis. The remaining part of the gene was cloned by nested PCR. Briefly, total DNA cut with a set of restriction endonucleases was ligated into the plasmid pBluescript II SK(+) with the same restriction enzymes. These ligation mixtures were used as PCR templates with gene-specific (P4 and P5) and vector-specific primers (T3 and SK). The PCR product was sequenced and primer P6 was designed based on the resulting DNA sequence. PCR for chromosome walking was performed under the following conditions: 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and polymerization at 72 °C for 3 min.

Chemical modification of bovicin HJ50. Chemical modification of bovicin HJ50 was performed according to Meyer et al. (1994). Bovicin HJ50 (8 μg) was dried under vacuum and resuspended in 50 μl of a modification mixture. The reaction mixture was incubated under nitrogen for 1 h at 50 °C followed by the addition of 2 μl acetic acid to stop the reaction. The reaction mixture was dried under vacuum and resuspended in water for MALDI-TOF MS analysis and peptide sequencing by Edman degradation.

Table 1. Primers used for cloning of bovA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GA(T/C)CGNGGNTGGAT(A/T/C)AA</td>
</tr>
<tr>
<td>P2</td>
<td>GA(T/C)AG(A/G)GGNTGGAT(A/T/C)AA</td>
</tr>
<tr>
<td>P3</td>
<td>AC(A/G)TTNGG(A/G)CA(A/G)TC(T/C)TT</td>
</tr>
<tr>
<td>P4</td>
<td>GGTTGATTAAGACCTTAAAC</td>
</tr>
<tr>
<td>P5</td>
<td>GAC(T/T)AACAAAGATGGCC</td>
</tr>
<tr>
<td>P6</td>
<td>TCTAACAGGTCTCTTGAGC</td>
</tr>
<tr>
<td>SK</td>
<td>CCGCTCTAGAACACTAGGGAT</td>
</tr>
<tr>
<td>T3</td>
<td>AXTTACCCCTCACTAAAGGG</td>
</tr>
</tbody>
</table>

Table 1. Primers used for cloning of bovA
RESULTS

Identification of the bovicin HJ50-producing strain

The bacteriocin-producing strain was a Gram-positive, amylase-positive, catalase-negative coccus able to grow at 45°C and at pH 9–6. However, it failed to grow at 10°C and in broth containing 6.5% NaCl. It gave a negative Voges-Proskauer reaction and produced no ammonia from the hydrolysis of arginine. The hydrolysis of aesculin was negative. It showed acid production from lactose, galactose and maltose, but not from inulin, mannitol, raffinose, ribose, salicin, sorbitol and trehalose.

16S rDNA analysis showed that the strain shares 99% homology with S. bovis NCD02127. Thus, the strain was named S. bovis HJ50.

Antimicrobial spectrum and susceptibility to proteases

As shown in Table 2, bovicin HJ50 was active against Lactobacillus curvatus LTH1174, Bacillus subtilis AS1.1087, Bacillus megaterium AS1.941, M. flavus NCIB8166, Leuconostoc dextranicum 181 and Leuconostoc mesenteroides AS1.2, but it showed no activity against Listeria monocytogenes. Like most other bacteriocins produced by LAB, bovicin HJ50 could only inhibit some strains of Gram-positive bacteria. Bovicin HJ50 could be inactivated by trypsin, subtilisin and proteinase K (data not shown).

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of strains tested</th>
<th>Zone of inhibition (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Bacillus coagulans AS1.949</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Bacillus megaterium AS1.941</td>
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<td>14</td>
</tr>
<tr>
<td>Bacillus subtilis AS1.1087</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>3</td>
<td>–</td>
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<tr>
<td>Lactobacillus acidophilus</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Lactobacillus brevis</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Lactobacillus buchneri AS1.40</td>
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<td>–</td>
</tr>
<tr>
<td>Lactobacillus curvatus LTH1174</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides AS1.2</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Leuconostoc dextranicum 181</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Micrococcus flavus NCIB8166</td>
<td>1</td>
<td>21</td>
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<tr>
<td>Pseudomonas aeruginosa 10105</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Shigella flexneri 51285</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Staphylococcus aureus 26071</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

*–, No inhibition.

Table 2. Antimicrobial spectrum of bovicin HJ50

Extraction, purification and characterization of bovicin HJ50

Like nisin and acidocin B (ten Brink et al., 1994), bovicin HJ50 could be extracted with n-propanol because of its hydrophobic properties. Most bovicin HJ50 remained in solution in n-propanol. In comparison with ammonium sulfate precipitation, the yield from n-propanol extraction was high (data not shown). After extraction, bovicin HJ50 was purified by ion exchange with SP Sepharose Fast Flow, followed by hydrophobic reaction chromatography with Phenyl Superose and gel filtration on Sephadex G-50.

Bovicin HJ50 increased the potassium permeability of M. flavus NCIB8166 in a concentration-dependent fashion (data not shown) and 20 AU bovicin HJ50 ml⁻¹ gave maximum potassium efflux.

MS (Fig. 1b) showed that the molecular mass of bovicin HJ50 was 3428 ± 3 Da, which was very close to the result of Tricine/SDS-PAGE (data not shown). The molecular mass of bovicin HJ50 reduced with DTT (Fig. 1a) was about 2–4 Da higher than that of untreated bovicin HJ50, indicating that bovicin HJ50 probably contains a disulfide bridge.

Edman degradation analysis revealed the N-terminal amino acid sequence of bovicin HJ50 to be Ala-Asp-Arg-Gly-Trp-Ile-Lys-X-Leu-X-Lys-Asp-X-Pro-Asn-Val-Ile-Ser-Ser-Ile. X at positions 8, 10 and 13 indicated blank cycles in which no

Fig. 1. MALDI-TOF mass spectrum of bovicin HJ50 treated with (a) and without (b) DTT.
Amino acid derivative was detected. Comparison of this sequence with sequences in the SWISS-PROT database showed that bovicin HJ50 is a novel peptide.

Analysis of the gene encoding bovicin HJ50

*bovA*, the structural gene of bovicin HJ50, was obtained by PCR using degenerate primers based on the N-terminal amino acid sequence, followed by nested PCR. As shown in Fig. 2, DNA sequencing confirmed the results of N-terminal sequencing of bovicin HJ50. The translation initiation site was arbitrarily assigned to the first of two ATG codons. *bovA* encodes a 58 aa prepeptide with a leader sequence of 25 aa. The leader peptide is hydrophilic, strongly charged and predicted to contain an α-helical conformation. It shows similarity with sequences of leader peptides of type AII lantibiotics (Fig. 3), including streptococcin A-FF22 (SA-FF22), lacticin 481, variacin (Priddle et al., 1996), mutacin II (Woodruff et al., 1998) and salivaricin A (Ross et al., 1993). It contains a GG (double-glycine) motif immediately preceding the cleavage site, and a conserved EL sequence (Sablon et al., 2000; Chen et al., 2001). The leader peptide of bovicin HJ50 contains a TVS motif instead of the conserved ETV/ESV sequences. The propeptide is a 33 aa peptide with a calculated mass of 3467-96 Da and a pl of 8-2. DNA sequencing revealed that the eighth and tenth amino acids of the bovicin HJ50 propeptide are both Thr and the thirteenth is Cys. The bovicin HJ50 propeptide also shows similarity with sequences of type AII lantibiotics (Fig. 3), especially salivaricin A. Propeptides of bovicin HJ50 and salivaricin A share 29-4% identity. Although bovicin HJ50 shows similarity with the sequences of type AII lantibiotics, the identity is low. The bovicin HJ50 propeptide is composed of 33 aa, whereas other AII lantibiotics range from 22 to 27 aa. The C-terminal sequence of bovicin HJ50 differs from that of type AII lantibiotics. Furthermore, the bovicin HJ50 propeptide contains four Cys residues, while other type AII lantibiotics contain only three.

A putative promoter is underlined in Fig. 2. The promoter contains a TG doublet 1 bp upstream of the −10 region (TATTAT). Approximately half of the lactococcal promoter sequences studied possess this characteristic (de Vos & Simons, 1994). In addition, downstream of *bovA*, a 20 bp palindrome may serve as a ρ-independent transcription terminator.

Chemical modification of bovicin HJ50

Chemical modification of lantibiotics has been used to determine the number of dehydrated amino acid residues in pep5, gallidermin (Meyer et al., 1994), mutacin I and mutacin III (Qi et al., 2000). To confirm that bovicin HJ50 contains modified amino acids, we performed an ethane-thiol modification of bovicin HJ50. Edman degradation analysis of ethanethiol-modified bovicin HJ50 revealed the
eighth and tenth amino acids were both \( \beta \)-methyl-S-ethylcysteine. However, no derivative was detected for the thirteenth residue. Two major peaks were generated after ethanethiol modification of bovicin HJ50 (Fig. 4). The two peaks showed molecular masses of 3490 and 3552 Da, respectively, which could be accounted for by bovicin HJ50 plus one or two molecules of ethanethiol. Our results revealed that Thr\(^8\) and Thr\(^{10}\) are modified, but probably none of the other Thr or Ser residues are modified.

**DISCUSSION**

In this study, we isolated a bacteriocin-producing strain from raw milk. Bacterial characters and phylogenetic analysis indicated that the bovicin HJ50-producing strain was *S. bovis*, a normal inhabitant of the cow rumen. Like most other bacteriocins produced by LAB, bovicin HJ50 is a small cationic peptide and could cause permeabilization of the target cell membrane. Bovicin HJ50 showed a narrow range of inhibitory activity.

Antibiotics are routinely fed to beef cattle in the USA to alter ruminal fermentation. As antimicrobial substances, bacteriocins produced by ruminal bacteria may have similar effects on ruminal fermentation. Several studies have been performed to investigate whether bacteriocins produced by *S. bovis* in the rumen have such an effect (Lee *et al.*, 2002, Mantovani *et al.*, 2002, Whitford *et al.*, 2001). Bovicin HC5 produced by *S. bovis* HC5 has a wide inhibitory spectrum and could inhibit a variety of freshly isolated *S. bovis* strains without causing adaptation. It is thought that it could be used to control the ruminal ecological environment. Further work is needed to investigate if bovicin HJ50 has a similar effect.

The evidence presented here shows that bovicin HJ50 is a lantibiotic. In lantibiotics, Ser, Thr and Cys are usually involved in the formation of unusual amino acids. In lantibiotic sequence analysis, Edman cleavage of a residue forming lanthionine or 3-methylanthionine would result in a blank cycle; however, the subsequent reactions would continue. Sequencing by Edman degradation is often blocked by a dehydro residue (Sahl *et al.*, 1995). Therefore, Thr\(^8\) and Thr\(^{10}\) may be involved in the formation of 3-methylanthionine with two Cys residues. Another two Cys residues form a disulfide bridge. Thus, bovicin HJ50 has two thioether bridges and a disulfide bridge. This would give a peptide with a calculated molecular mass of 3429-96 Da. This value was in a good agreement with the molecular mass of 3428-3 Da obtained by MS of bovicin HJ50.

MS analysis of bovicin HJ50 reduced with DTT indicated that bovicin HJ50 contains a disulfide bridge. However, when assayed in the presence of DTT, the titre of bovicin HJ50 against *M. flavus* NCIB8166 was neither decreased nor increased (data not shown). Lantibiotics containing a disulfide bridge are an anomaly in the bacteriocin world. To our knowledge, only sublancin 168 produced by *Bacillus subtilis* 168 (Paik *et al.*, 1998) and plwz produced by *Lactobacillus plantarum* LMG 2379 (Holo *et al.*, 2001) contain disulfide bridges.

At present most of the bacteriocins produced by *Streptococcus* strains are lantibiotics, such as salivaricin A, SA-FF22, mutacin I (Qi *et al.*, 2001), mutacin II and mutacin III (Qi *et al.*, 1999), etc., while bovicin 255 and mutacin IV are regarded as non-lantibiotics. Bovicin HJ50 is a lantibiotic with the unusual characteristic that it contains a disulfide bridge.

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Fig. 4. MALDI-TOF mass spectrum of ethanethiol-modified bovicin HJ50.
REFERENCES


