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Purification of bacteriocin LS1 produced by human oral isolate *Lactobacillus salivarius* BGHO1

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Introduction: *Lactobacillus salivarius* BGHO1, a human oral isolate with antagonistic activity against growth of *Streptococcus mutans*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Micrococcus flavus*, and *Salmonella enteritidis*, probably produces more than one proteinaceous antimicrobial substance. The objective of this study was the purification of a bacteriocin, named LS1, produced by *L. salivarius* BGHO1.
Methods: A simple and fast procedure for bacteriocin purification was developed, consisting of reverse-phase chromatography of the ammonium sulfate precipitate of cell-free culture supernatant by fast protein liquid chromatography and high-performance liquid chromatography, followed by tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), with the subsequent extraction of bacteriocin from the gel.
Results: The supernatant of *L. salivarius* BGHO1 culture retained its antimicrobial activity after boiling in a water bath for 15 min. Its antimicrobial activity was also maintained even after treatment for 20 min at 121°C in an autoclave. Bacteriocin LS1 was purified to homogeneity. The molecular mass of bacteriocin LS1 was estimated to be approximately 10 kDa, based on tricine SDS-PAGE. During purification, another compound with antimicrobial activity, produced by *L. salivarius* BGHO1, was detected. The molecular mass of this compound was estimated to be approximately 5 kDa, based on tricine SDS-PAGE.
Conclusion: Our results imply that LS1 is most probably a new bacteriocin, different from previously described bacteriocins produced by *L. salivarius* strains. The purification of bacteriocin LS1 enabled the further characterization of LS1 on both the molecular and genetic levels.

Key words: bacteriocin; chromatography; human oral isolate; *Lactobacillus salivarius*

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Bacteriocins produced by lactic acid bacteria are a heterogeneous group of antibacterial proteins (12). Most bacteriocins have narrow inhibitory spectra (4). Only a few of them have wider inhibitory spectra, being active against a wide range of bacteria (1, 6, 7). They exert their activity through adsorption to specific target molecules located on the external surface of sensitive bacteria, followed by metabolic, physiological, and morphological changes resulting in the death of the targeted bacteria (8). Major classes of bacteriocins produced by lactic acid bacteria include: (class I) lantibiotics, (class II) small heat-stable peptides, (class III) large heat-labile proteins, and (class IV) complex proteins whose activity requires the association of carbohydrate or lipid moieties (8, 12).

Lactobacilli isolated from different ecological niches, such as the oral cavity, could be a source of new bacteriocin-producing probiotics. Probiotic microflora display numerous health benefits, such as the competitive exclusion of medically significant pathogens and the treatment and neutralization of side effects of antibiotic therapy (10, 11). In addition, they maintain cooperatively a delicate balance between the gastrointestinal tract and the immune system (3). One of the desirable properties of a probiotic strain is

its ability to produce antimicrobial substances such as bacteriocins (9). In a previous study, we made a collection of lactobacilli isolated from different locations in the human oral cavity with the objective of finding strains possessing potential probiotic activities and characterizing their properties, such as antimicrobial activity and resistance to bile salts and low pH (14). The strain *Lactobacillus salivarius* BGHO1 was selected for further study as a potential producer of more than one antimicrobial substance of proteinaceous nature with antagonistic activity on the growth of *Streptococcus mutans*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Micrococcus flavus*, and *Salmonella enteritidis*, and also on some non-pathogenic bacteriocin-producing laboratory strains of lactic acid bacteria. Moreover, the isolate *L. salivarius* BGHO1 was tolerant of the low pH and high bile-salt concentrations, which are limiting conditions for passage through the gastrointestinal tract (14).

According to our knowledge, only two bacteriocins from *L. salivarius* isolates have been purified; their primary structure has been characterized and one of them was also characterized genetically (5, 13). In this study, we describe the purification of the bacteriocin termed LS1, produced by the human oral strain *L. salivarius* BGHO1. For this purpose, a simple and fast bacteriocin purification procedure was applied. It consisted of reverse-phase (RP) chromatography steps on fast protein (FPLC) and high-performance (HPLC) liquid chromatography systems, followed by tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), with the subsequent extraction of bacteriocin from the gel.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *Lactobacillus* strains were grown in liquid MRS broth (Merck, Darmstadt, Germany) or on solid MRS agar plates [1.5% weight/volume (w/v)] at 37°C for 16 h. *Lactococcus lactis* and *S. mutans* strains were grown in liquid M17 broth (Merck) containing 5 g/l glucose (GM17 medium) or on solid GM17 agar plates (1.5% w/v), at 30 and 37°C, respectively, for 16 h. *Lactobacillus salivarius* BGHO1 and *S. mutans* BGSF1 were grown under quiescent aerobic conditions.

Antimicrobial activity assay

For detection of antimicrobial activity, an agar-well diffusion assay was used (15). Aliquots (50 µl) of fresh overnight culture of *L. lactis* subsp. *cremoris* NS1 or *S. mutans* BGSF1, were added to 20 ml soft GM17 agar (0.8%, w/v) and poured into sterile Petri dishes. Wells were made in the lawn of hardened GM17 soft agar. Aliquots (100 µl) of fresh overnight cultures (16 h), or concentrated fractions from different steps of purification (see section on Bacteriocin purification) were poured into wells. Plates inoculated with the indicator strains *L. lactis* and *S. mutans* were incubated for 16 h at 30 and 37°C, respectively. After 16 h of incubation, inhibition zones were scored. To test the fractions collected during RP chromatography on HPLC, the assay was modified as follows: instead of making wells, drops of 10 or 20 µl were applied directly to the surface of soft agar inoculated with the indicator strain.

Temperature stability of the bacteriocin

To study the thermostability of the bacteriocin, 50-µl aliquots of concentrated supernatant from overnight cultures of *L. salivarius* BGHO1 were boiled in a water bath for 15 min, subsequently cooled to room temperature and assessed for their antimicrobial activity using the agar-well diffusion assay described above.

Bacteriocin purification

Purification of bacteriocin LS1 from the strain *L. salivarius* BGHO1 involved ammonium sulfate precipitation, followed by RP chromatography on FPLC and HPLC systems with the final purification step by tricine SDS-PAGE.

Table 1. List of strains used in this study

Strains	Relevant characteristics[1]	Source or reference
Lactobacillus salivarius BGHO1	Bac^+, Bac^{Im}	(14)
Lactococcus lactis subsp. *cremoris* NS1	Bac^-, Bac^s	Laboratory collection
Streptococcus mutans BGSF1[2]	Clinical isolate, Bac^s	(14)

[1]Bac^+, Bac^{Im} – bacteriocin producer, bacteriocin immunity; Bac^-, Bac^s – bacteriocin non-producer, bacteriocin sensitive.
[2]Identified by sequencing of the 16S rDNA gene.

Broth culture of *L. salivarius* BGHO1 was grown in 700 ml of MRS medium at 37°C for 16 h under quiescent aerobic conditions. Cultures were centrifuged at 6000 ***g*** for 20 min to remove cells. The supernatant was adjusted to pH 6.4 and submitted to ammonium sulfate fractionation.

The ammonium sulfate precipitate was fractionated by chromatography. The 60% (w/v) fraction was resuspended in 50 mM sodium phosphate buffer pH 6.4 and diluted 10-fold in 0.11% (v/v) trifluoroacetic acid (TFA) water solution and loaded on an RP column R1 Poros (Applied Biosystems, Foster City, CA; 1.5 × 15 cm), equilibrated with 70% (v/v) solvent A (0.11%, v/v, TFA), 30% solvent B [0.09% TFA, 85% acetonitrile (ACN), 5% isopropanol], on Bio Cad FPLC. Bacteriocin was eluted from the column using a 40-min linear gradient of 30–100% solvent B. The flow-rate was 6 ml/min and the absorbance was recorded at 220 and 280 nm. Fractions (10-ml) were collected, concentrated by Speed Vac (Model SC110A; Savant Instrument Inc. Farmingdale, NY) and tested for antimicrobial activity. Active fractions were kept at +4°C. Bacteriocin was further purified by RP-HPLC using a Waters Alliance apparatus with MILLENNIUM software (Waters, Millford, MA). Aliquots of 100 µl active fraction were repeatedly injected into analytical C_{18} Waters Symmetry column (300 Å, 5 µm, 3.9 × 150 mm), until the column was saturated with sample. The column was equilibrated with 0.11% (v/v) TFA in MilliQ (Millipore, Bedford, MA) water (solvent C). Elution was performed using 40-min two-part linear gradient: 0–50% (v/v) of solvent D (0.09% TFA, 85% ACN, 5% isopropanol in MilliQ water) for 10 min and 50–100% (v/v) of solvent D in 30 min. Flow rate was 0.8 ml/min. Eluted peaks were detected by measuring the absorbance between 210 and 300 nm with a photo-diode detector (PDA 996; Waters, Millford, MA). Fractions were collected and concentrated in a Speed Vac (Model SC110A, Savant Instrument, Farmingdale, NY).

Fractions with antimicrobial activity were dried, resuspended in 10 µl 20 mM sodium phosphate buffer pH 6.4, mixed with 10 µl sample buffer with β-mercaptoethanol, boiled for 5 min, and then run on 16.5% acrylamide gel for tricine SDS-PAGE on a Bio-Rad electrophoresis system, together with polypeptide standard (Bio-Rad, Hercules, CA). Gels were stained with Coomassie blue G250, according to the protocol supplied with the Bio-Rad polypeptide standard. Protein

bands were excised from the gel and transferred into 1.5-ml tubes. Protein elution from the gel was performed as described previously (2) with some modifications: the gel slice was crushed into small pieces and overlaid with elution solution composed of 0.09% TFA, 50% ACN, in MilliQ water. The tube was closed and stirred at room temperature for 12 h. After centrifugation, solution was concentrated in a Speed Vac and tested for antimicrobial activity.

Verification of the proteinaceous nature of the bacteriocin

The proteinaceous nature of the antimicrobial activity of the sample used for RP-HPLC was tested. The sample was incubated with proteinase K (Merck), at a final concentration of 1 mg/ml, in the buffer recommended by the manufacturer. As controls, enzyme alone in the buffer and sample in the buffer without enzyme were used. After 2 h of incubation at 37°C, the reaction was stopped by heating at 100°C for 5 min, subsequently cooled to room temperature spontaneously, and assayed for antimicrobial activity using *L. lactis* subsp. *cremoris* NS1, as indicator strain.

Results

Antimicrobial activity of the supernatant of *L. salivarius* BGHO1 culture against *L. lactis* subsp. *cremoris* NS1 and *S. mutans* BGSF1 indicators was stable after boiling in a water bath for 15 min. Antimicrobial activity against both indicators was retained even after treatment in an autoclave for 20 min at 121°C. The proteinaceous nature of antimicrobial activity present in the supernatant of *L. salivarius* BGHO1 culture was confirmed previously (14). After ammonium sulfate precipitation, antimicrobial activity against *L. lactis* subsp. *cremoris* NS1 and *S. mutans* BGSF1 indicators was observed only in the fraction of 60% ammonium sulfate saturation. This fraction was applied on RP-FPLC.

Two distinct zones along the elution range showed antimicrobial activity. Both fractions were eluted after a main peak, which probably accounted for the majority of compounds with an average hydrophobicity (Fig. 1). The active fractions obtained from these two samples were collected together and used as one sample for the next purification step (RP-HPLC on C_{18} column). Before performing RP-HPLC, the proteinaceous nature of the antimicrobial activity of this sample was confirmed (data not shown).

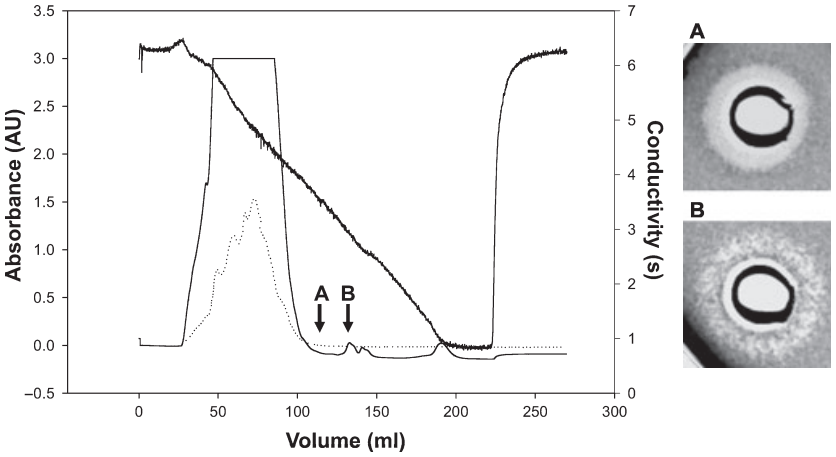

Fig. 1. Chromatogram of reverse-phase chromatography on a fast protein liquid chromatography R1 column of 60% ammonium sulfate precipitate of *Lactobacillus salivarius* BGHO1 culture supernatant in MRS broth. Arrows indicate fractions with inhibitory activity. Absorbance was measured at 220 nm (black line) and 280 nm (dotted line). Corresponding zones of inhibition from the antimicrobial activity assay with *Lactococcus lactis* subsp. *cremoris* NS1 as indicator strain are given on the right side of the figure.

With the better resolution of the HPLC system and the ability to examine the activity of the collected fractions, two fractions with antimicrobial activity were precisely localized, close to each other and eluted after a main peak (Fig. 2). Fraction A, which produced a larger zone of inhibition, was eluted at 52.8% of elution buffer and fraction B, with the smaller inhibition zone, was eluted 4 min later at 58.3% of elution buffer. These values represent the percentage of the buffer that enters into the detector, calculated on the basis of time between injection and the moment when unbound fraction reaches the detector. This step of purification was performed five times, to confirm the reproducibility of the purification method and to collect as much as possible of the sample for the final step of purification, which consisted of tricine SDS-PAGE. Active fractions A and B obtained from RP-HPLC were applied on 16.5% acrylamide tricine SDS-PAGE. In the case of fraction A, a band of approximately 10 kDa was observed (Fig. 3, lane 5). In the case of fraction B, a weak band of approximately 5 kDa was observed when stained with Coomassie blue G-250 (data not shown). Both bands were excised from

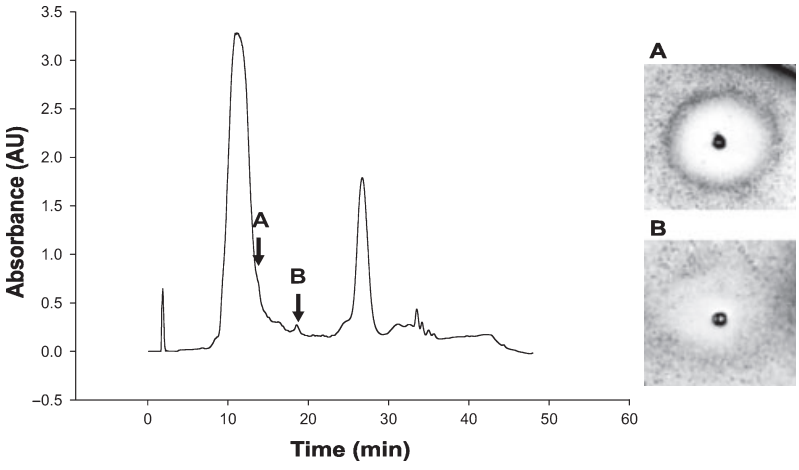

Fig. 2. Chromatogram of reverse phase (RP) chromatography on high-performance liquid chromatography on a C_{18} column of active fractions obtained from RP-fast protein liquid chromatography of 60% ammonium sulfate precipitate of *Lactobacillus salivarius* BGHO1 culture supernatant grown in MRS broth. Arrows indicate fractions with inhibitory activity. Absorbance was measured at 220 nm and corresponding zones of inhibition from antimicrobial activity assay with *Lactococcus lactis* subsp. *cremoris* NS1 as indicator strain are shown on the right side of the figure.

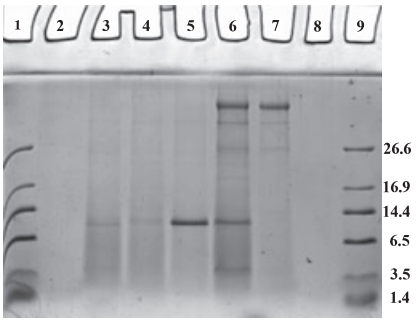

Fig. 3. Analysis of fractions eluted from reverse-phase high-performance liquid chromatography resolved on tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Lane 5, active fraction A; lanes 3 and 4, neighboring fractions eluted before the active fraction A; lanes 6 and 7, neighboring fractions eluted after active fraction A; lanes 1 and 9, polypeptide SDS-PAGE molecular weight standard (Bio Rad). The gel was stained with Coomassie blue G-250.

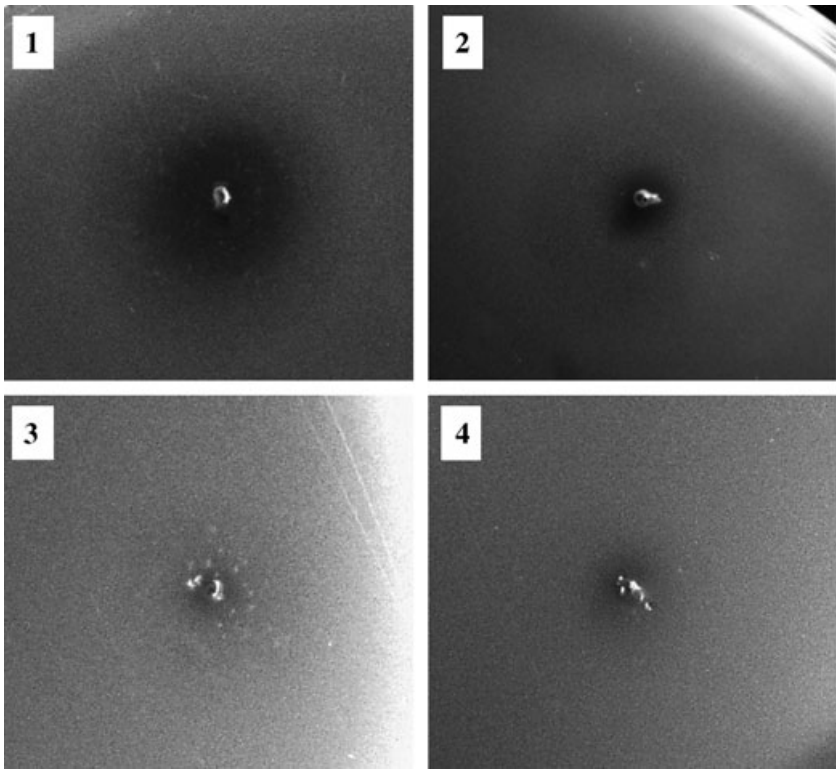

Fig. 4. Antimicrobial activity assay with *Lactococcus lactis* subsp. *cremoris* NS1 as indicator strain. Sample 1, 10 μl eluate from the gel slice containing 10-kDa band, with visible zone of antimicrobial activity; sample 2, 10 μl eluate from the gel slice containing 5-kDa band, with an additional small but visible zone of antimicrobial activity; sample 3, 10 μl eluate from the slice of the same gel without any visible band, control for samples 1 and 2; sample 4, 10 μl eluate from the gel slice containing the 16.95-kDa myoglobin band from polypeptide standard (Bio-Rad), control for samples 1 and 2.

the gel and proteins were extracted. The proteins extracted from these bands were assayed for antimicrobial activity, together with appropriate controls (Fig. 4). Controls represent eluate from the slice of the same gel that did not contain any visible band and from the gel slice with myoglobin band from the polypeptide standard (Bio-Rad). Antimicrobial activity was observed in eluate from the gel slice containing an *c*. 10-kDa band obtained from fraction A of RP-HPLC. A small but visible zone of antimicrobial activity was also observed in the eluate of the gel slice containing the *c*. 5-kDa band obtained from fraction B of RP-HPLC. Bacteriocin extracted from the *c*. 10-kDa band was designated bacteriocin LS1.

Discussion

To develop a simple and fast procedure for the purification of bacteriocins, we combined RP chromatography steps of ammonium sulfate precipitate on FPLC and HPLC systems with tricine SDS-PAGE, with subsequent extraction of the resolved bands. In this way, we avoided steps that needed to be standardized such as ion-exchange chromatography, after which, because of desalting, samples may lose part of their antimicrobial activity. In addition, to examine the antimicrobial activity of particular bands on the gel, eluted protein from excised gel bands was used instead of the classical method of overlaying the gel with soft agar medium inoculated with indicator strain. The classical method needs to be standardized, because of frequent problems of indicator inhibition by the gel or problems occurring during rinsing steps, such as bacteriocin diffusion from the gel.

Fractionation of the supernatant of *L. salivarius* BGHO1 culture by precipitation with a 60% saturation of ammonium sulfate gave one fraction with antimicrobial activity. RP chromatography was chosen as a simple, fast step of purification with good separation. The fact that, after the RP chromatography step, fractions do not need to be treated too much for the subsequent antimicrobial assay, represents an important aspect of our method. Samples should only be concentrated and after pH adjustment, their antimicrobial activity can be directly tested.

To obtain a better resolution of separation, active fractions of RP-FPLC were collected and mixed together into one sample for the next step of RP-HPLC.

Until now, only two bacteriocins from different *L. salivarius* isolates have been purified and characterized (5, 13). The approximate molecular mass of bacteriocin LS1, deduced from molecular weight standard on tricine SDS-PAGE (*c*. 10 kDa for post-RP-HPLC fraction with inhibitory activity), suggests that LS1 is most probably a new bacteriocin, different from the two that were previously described [molecular mass of bacteriocin ABP-118, a class IIb bacteriocin, is 4096.69 Da (5) and that of bacteriocin OR-7, a class IIa bacteriocin, is 5132 Da (13)]. Further research, encompassing mass spectrometry and N-terminal sequencing, will give more precise information on the exact molecular mass of bacteriocin LS1 and its partial amino acid sequence.

Acknowledgments

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