Characterization and purification of a bacteriocin-like substance produced by *Lactobacillus crispatus* LBS 17-11 isolated from an oral cavity of human subject

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Purpose: *Lactobacillus crispatus* LBS 17-11 was isolated from an oral cavity of human subject, and possess growth inhibition activity against *Streptococcus pneumoniae*. The objective of this study was to purify a bacteriocin-like substance, and to determine its characteristic properties.
Materials and Methods: Antibacterial activities against *S. pneumoniae* ATCC 33400 during purification steps and against several oral bacteria were determined by a radial diffusion assay. Purification procedure for bacteriocin-like substance was achieved with ammonium sulfate precipitation of culture supernatant, and subsequent extraction with chloroform. Minimum inhibitory concentration (MIC) was determined by liquid microdilution method.
Results: The culture supernatant of *L. crispatus* LBS 17-11 showed a bacteriostatic activity against *S. pneumoniae*. This substance was partially purified up to 2045-fold specific activity (unit/mg protein). The inhibitory activity was shown against only *S. pneumoniae*, but Gram negative species. The antimicrobial activity was heat stable and partially lost by the treatment with proteases (trypsin and proteinase K). The molecular mass of bacteriocin-like substance was estimated to be approximately 7 kDa based on tricine SDS-PAGE. Binary combination of 1/2-MIC level of sodium lactate (56 mM) and the bacteriocin-like substance showed lower MIC level compared to that of the addition of the bacteriocin-like substance alone.
Conclusion: Bacteriocin-like substance produced by *L. crispatus* LBS 17-11 showed bacteriostatic activity against *S. pneumoniae*. The partially purified bacteriocin-like substance was heat stable and degraded by proteases. Molecular mass was estimated to be approximately 7 kDa.

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Key Words: bacteriocin, *Lactobacillus crispatus*, probiotics, *Streptococcus pneumoniae*

Introduction

Bacteriocin is natural antibacterial peptides ribosomally biosynthesized by bacteria. The proteinaceous nature of bacteriocin is shown to be degraded easily, and not to remain in human body. Therefore, they are recognized as a safer conventional food preservation [1,2]. Various bacteriocins have been identified in Gram positive bacteria, including lactic acid bacteria. They are classified into two groups, class I and class II, and subclasses based on its structure [1,2]. Nisin is classified as a Type I lantibiotic and containing several unusual amino acids. Class II bacteriocins, nonlantibiotic bacteriocins, are further divided into four subclasses. Class IIa shows anti-*Listeria* activity. Class IIb bacteriocins consist of two peptides and Class IIc bacteriocins are circular peptide and remaining other bacteriocins are classified as Class IId. All bacteriocins shows heat stable nature.

Oral lactobacilli were previously believed to be the cariogenic microorganism because of their high numbers in carious lesions and their acidogenic properties. Subsequent research has shown that they are associated with the caries progression in dentine rather than with the caries initiation in the enamel [3,4]. Lactobacilli are also believed to be important organisms for modification of the flora in the human body based on their ability to suppress growth of pathogens [5]. One of the main mechanisms for *Lactobacillus* strain to inhibit growth of pathogens is a production of antimicrobial substances, such as organic acid, hydrogen peroxide and bacteriocins [4, 6-10].

In our laboratory, we have been investigating the bacteriocins isolated from oral microorganisms for the application to oral health, such as prevention of dental caries, periodontal diseases and infection of respiratory organs [11]. Several oral bacteria are thought to cause systemic diseases, such as bacteremia, endocarditis, pneumonia and sepsis. *Streptococcus pneumoniae* is one of pathogens involved in upper respiratory tract infections in humans [8,12,13]. Therefore, it is important to eliminate oral pathogens for health. In this study, we investigated the purification of bacteriocin-like substance produced by *L. crispatus* LBS 17-11, which was isolated from an oral cavity of human subject, and determined its characteristic property of the bacteriocin-like substance against *S. pneumoniae*.

Materials and Methods

Chemicals

Trypsin, Proteinase K and sodium lactate (50%) were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Nisin was purchased from MP Biomedicals, LLC (Solon, OH, USA) and used as a comparative control.

Bacterial strains and culture conditions

S. pneumoniae ATCC 33400, *Streptococcus mutans* ATCC 25175^T, *Streptococcus sobrinus* ATCC 33478^T, *Fusobacterium nucleatum* JCM 8532^T, *Prevotella intermedia* ATCC 25261^T, and *Porphyromonas gingivalis* ATCC 33277^T were the stock culture collection of Tsurumi University (Yokohama, Japan). Each strain was inoculated into Triptic-Soy broth (TSB; Becton, Dickinson & Co. Detroit, MI, USA) and incubated in anaerobox (Concept Mini, Ruskinn, Central Scientific Commerce, Inc., Tokyo, Japan) under an atmosphere comprising $N_2/CO_2/H_2$ (80:10:10) at 37˚C for 24-48 hours. *P. gingivalis*, *P. intermedia*, and *F. nucleatum* were incubated in TSB supplemented with 1 μg/mL of menadione and 5 μg/mL of hemin and incubated at 37˚C for 48 hours under anaerobic condition.

Determination of bacteriocin-like activity

L. crispatus LBS 17-11 was cultured in Lactobacilli; MRS Broth (MRS; Becton, Dikinson Co.) for 24 hours. The culture broth was then centrifuged at 1,710 × *g* for 30 minutes. Antibacterial activities against *S. pneumoniae* ATCC 33400 during purification steps and against several oral bacteria were determined by a radial diffusion assay. Aliquots (100 μL) of overnight culture (16 hours) of *S. pneumoniae*, as the indicator strains, were mixed into 10 mL TS agar (1.0% agar, w/v) and poured into sterile Petri dishes (85 mm diameter). Aliquots of fresh overnight cultures or concentrated fractions (5-10 μL) from different steps of purification were poured into wells. Plates inoculated with *S. pneumoniae* were incubated for 16 hours at 37˚C, and then inhibition zones were scaled.

Minimum inhibitory concentration (MIC) was determined by liquid microdilution method in accordance with the Standard Method of the Japan Society of Chemotherapy (1990) with minor modification. A 20 μL of two-fold serial dilution of samples was put into microtiter wells. Bacterial suspensions adjusted to absorbance turbidity. Then, 200 μL of these bacterial suspensions was added to 20 mL of the assay media, 180 μL of aliquots were put into microtiter plate wells. The culture was incubated anaerobically for 16-24 hours at 37˚C. MIC was defined as the lowest concentration for inhibiting visible growth.

Purification of bacteriocin-like substance

One liter of the supernatant of *L. crispatus* LBS 17-11 was precipitated with 35-80% ammonium sulfate with

stirring. The precipitated proteins were collected by centrifugation of 1,710 × *g* at 4˚C for 40 minutes and then resuspended in 10 mL of phosphate buffered saline (PBS; pH 7.0). The precipitated proteins were dialyzed twice against one L of PBS for 24 hours using a dialysis bag. The dialyzed proteins were mixed violently with an equal volume of chloroform and then centrifuged with 1,710 × *g* at 4˚C for 30 minutes. Remaining precipitated proteins was also extracted an equal volume of chloroform. The total organic phase was evaporated using a rotary evaporator.

Determination of protein concentration and tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

Protein content was estimated using Takara BCA Protein Assay Kit (Takara Bio Inc., Kusatsu, Japan), according to manufacturer's instruction. Tricine-SDS PAGE were performed with 15% acrylamide gel (e-PAGEL, ATTO Corp. Tokyo, Japan). The gel was cut into two parts; one was stained with EzStain (AE-1340 EzStain AQua, ATTO Corp.), the other was rinsed in 0.1 % Triton X100 in sterile water for 30 minutes, followed by three times rinsing in sterile water for 30 minutes, finally rinsed in PBS for 30 minutes. The gel was overlaid on test agar plate include *S. pneumoniae* indicator, and incubated in anaerobic condition at 37˚C for 16 hours.

Characterization of bacteriocin-like substance

The heat stability and proteinase sensibility were investigated. The partially purified protein (120 μg/mL) obtained by final step and proteinase K (1 mg/mL) or trypsin (1 mg/mL) in PBS were incubated at 37˚C for 3 hours. Remained activity was measured by the diameters of inhibition zone of a radial diffusion assay, and percentage of remained activity was calculated by a standard curve on semi-logarithmic plot of diameter-dose responses.

Results

Purification of the bacteriocin-like substance of *L. crispatus* LBS 17-11

Indicator strain was *S. pneumoniae* ATCC 33400 for purification of this substance. Bacteriocin-like activity was found in the supernatant of overnight culture of *L. crispatus* LBS 17-11. The growth inhibition activity showed a peak in the supernatant at 16 hours bacterial culture and its activity was lasting for more 2 days (data not shown). The strong antimicrobial activity was recovered in the precipitation of 35 to 80% of ammonium sulfate. The recovery of protein from culture supernatant was 5.2%, and purification of antimicrobial activity was 15-fold increased. The ammonium sulfate precipitated protein was dialyzed and further extracted with chloroform. The purification of antimicrobial activity was up to 2045-fold increased, and its recovery of activity was 19% of initial culture supernatant (Table 1).

Table 1 Purification stage and antimicrobial activity of *L. crispatus* LBS 17-11

Purification step	Volume (mL)	Activity (unit/mL)	Total protein (mg)	Protein recovery (%)	Purification (fold)	Recovery of activity (%)
Culture supernatant	1,000	3	7,751	100	1	100
Ammonium sulfate precipitation (35-80%)	40	60	404	5.2	15	80
Chloroform extraction	6	95	0.72	0.01	2,045	19

Characterization of the bacteriocin-like substance of *L. crispatus* LBS 17-11

Anti-microbial activity against *S. pneumoniae* was observed, but this activity was bacteriostatic. Slight visible

bacterial cells were observed in a clear zone of a radial diffusion assay, and in the well of higher concentration than MIC in a liquid microdilution assay. *S. mutans*, *S. sobrinus*, and Gram negative bacteria, such as *P. gingivalis*, *P. intermedia*, and *F. nucleatum* were not sensitive to this substance using a radial diffusion assay. The only one band was found to be molecular mass of approximately 7 kDa based on Tricine-SDS-PAGE (15%) with a low range molecular weight standard. The Ez-stained band coincident with the bacteriostatic spot of agar plates inoculated with *S. pneumoniae* seemed to be high purified bacteriocin-like substance (Fig. 1). The antimicrobial activity was heat stable at 100˚C for 10 minutes. The characteristic of this substance showed proteinaceous nature, because the activity was partially lost (60 or 55% of control) by the treatment of trypsin or proteinase K, respectively (Table 2).

Effect of combined use of the bacteriocin-like substance and sodium lactate

Because anti-microbial activity against *S. pneumoniae* was bacteriostatic, we determined the combination of this substance and sodium lactate, which is recognized as safe compound for food preservation. Nisin, which is a bacteriocin produced by a group of Gram-positive bacteria that belongs to *Lactococcus* and *Streptococcus* species, was used as comparative control. Addition of 1/2-MIC level of sodium lactate (56 mM) and the bacteriocin-like substance showed lower MIC level compared to that of the addition of the bacteriocin-like substance alone. The combination effect of nisin and sodium lactate was not observed (Table 3).

Table 2 Effect of protease and heat treatment on partially purified bacteriocin-like activity

Treatment	Antimicrobial activity (%)
Control	100
Trypsin 1 mg/mL, 3 hours	60
Proteinase K 1 mg/mL, 3 hours	55
100˚C, 10 minutes	100

Table 3 Effect of combination of sodium lactate with bacteriocin-like substance and nisin against *S. pneumoniae*

	MIC	
Sodium lactate	112	mM
Bacteriocin-like substance (BLS)	1.5	μg protein /mL
Nisin	0.63	μg protein /mL
BLS + Sodium lactate (56 mM)	0.375	μg protein /mL
Nisin + Sodium lactate (56 mM)	0.63	μg protein /mL

MIC was determined by liquid microdillution method against *S. pneumoniae* ATCC 33400.

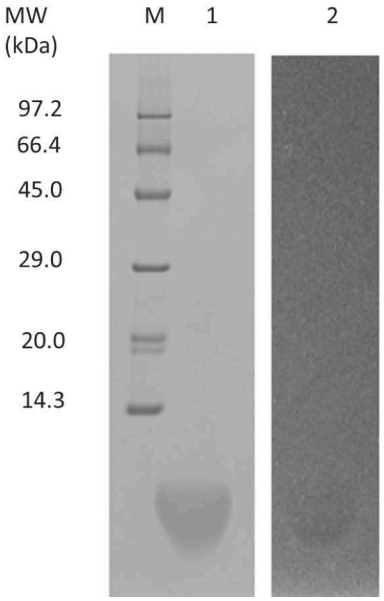

Fig. 1

Tricin-SDS PAGE analysis of partial purified bacteriocin-like substance from *Lactobacillus crispatus* LBS 17-11. Lanes: M, molecular mass standard (ATTO AE-1440 EzStandard) with sizes given on the left. Lane 1: chloroform extracted bacteriocin-like substance stained with EzStain. Lane 2: gel overlaid with 1% agar plate containing *S. pneumoniae* ATCC 33400.

Discussion

In this study, we reported on purification of the bacteriocin-like substance against *S. pneumoniae* produced by *L. crispatus* LBS 17-11, which was isolated from an oral cavity of human subject. The definition of the bacteriocin is an antimicrobial peptide and proteins ribosomally synthesized by bacteria. All bacteriocins are heat stable [1]. In the present study, the bacteriocin-like substance was heat stable, and showed proteinaceous nature, because activity was partially lost by the treatment of trypsin or proteinase K. However, the stained band in the Tricin-SDS-PAGE was disappeared gradually. Strangely, this substance did not show bactericidal activity, but

show bacteriostatic activity against *S. pneumoniae*. The growth of the other oral bacteria, Gram positive bacteria such as *S. mutans* and *S. sobrinus,* and Gram negative bacteria, such as *P. gingivalis*, *P. intermedia* and *F. nucleatum* were not inhibited. This property was similar to bacteriocin, but it was slightly different from previously described definition of bacteriocin. Therefore, we thought this substance was "bacteriocin-like" and "partial purification substance".

In the purification procedure, the chloroform extraction of the ammonium precipitated protein increased the antimicrobial activity, suggesting a high degree of purification of this substance. It may be explained by that some bacteriocins appear in their native state as aggregates with other molecules such as lipid materials. These aggregates may mask the antimicrobial activity partially. Such complexities can be eliminated by extraction with chloroform. Contreras et al. demonstrated that a simple one-step methanol-chloroform extraction could remove most fatty acid contamination from the ammonium sulfate precipitated bacteriocin, which resulted in a pure bacteriocin [14]. Wannun et al. also reported the purification of bacteriocin produced by oral *Lactobacillus paracasei* SD1 by using the chloroform extraction method. In the present study, the bacteriocin-like substance was extracted with chloroform, which indicated this substance has a hydrophobic character [9].

Generally, the bacterium is pH sensitive, and its growth rate decreased at acidic pH. Usually, the screening method of probiotic candidate was performed after adjustment of culture supernatant with NaOH to eliminate a bactericidal effect by organic acid. However, sodium lactate was potent growth inhibitor, as well as sodium citrate against *S. pneumoniae* and several oral bacteria [15]. Moreover, the synergistic action of nisin and sodium lactate on the growth of *Listeria innocua* and *Shewanella putrefaciens* were reported, previously [16].

S. pneumoniae is prevalent in the oral cavity and/or the upper respiratory tract and it is a leading cause of pneumonia, meningitis and septicemia in humans [12]. The nature of bacteriostatic substance may not be suitable for the application for the prevention of pneumococcal diseases, directly. Ooshima *et al*, reported that the mutacin, which was the bacteriocin produced by *S. mutans*, completely killed the cells grown in sucrose-free medium, whereas the bacteriocin did not kill the *S. mutans* cell grown in a sucrose-containing medium [17]. The inhibitory mechanism of the bacterioci-like substance in this study may be different from that of other known bacteriocins. Some papers described that bacteriocins may have a function as signaling peptides, either signaling other bacteria through quorum sensing and bacterial cross talk within microbial communities or signaling cells of the host immune system [18,19].

The partial purification of bacteriocin-like substance enable the further purification and bacteriostatic characterization may contribute to an analysis of oral bacterial ecosystems, and may be an alternative approach for promoting oral health or prevention of oral diseases.

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Conflict of Interest
N. Hanada and S. Imai received research fund from Yakult Central Institute (Tokyo, Japan). All remaining authors declare no potential conflicts of interest with respect to authorship and publication of this article.

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