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Characterization of Buchnericin LB Produced by Lactobacillus buchneri LB

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Abstract: *Lactobacillus buchneri* LB isolated from a commercially fermented vegetable product produced a bacteriocin-designated buchnericin LB. It was found that buchnericin LB was heat-stable (90-121°C for 15 min) and hydrolyzed by proteases, α-chymotrypsin, trypsin, papain, proteinase K and pepsin. However, it was observed that it was resistant to catalase, peroxidase, lipase, amylases and organic solvents. Also, it was found that it retained its biological activity after exposure to pH 2.0-9.0 and after storage at -20° and -70°C. It was determined that buchnericin LB had a wide inhibitory spectrum, being inhibitory to the *Listeria, Bacillus, Enterococcus, Micrococcus, Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus* species tested, and its molecular weight was determined to be about 3.5-4.5 kDa by tricine-SDS-PAGE.

Key Words: Bacteriocin, buchnericin LB, *Lactobacillus buchneri* LB.

Introduction

Bacteriocins are proteinaceous compounds produced by bacteria that exhibit a bactericidal or bacteriostatic mode of action against sensitive bacterial species (1, 2). Within the past several years there has been an increased interest in the development of bacteriocins as food preservatives. The authorization by the U.S. Food and Drug Administration (FDA) in 1988 for the use of nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, as a food biopreservative in pasteurized processed cheese spreads opened the door for the potential application of bacteriocins in foods. Its effectiveness as a processing aid has been demonstrated in many products worldwide for over 40 years. With the precedent use of nisin in foods, the potential exists for the use of other bacteriocins as effective food preservatives. The production of bacteriocins from lactic cultures potentially shortens the regulatory process because lactic acid bacteria essentially have GRAS (generally recognized as safe) status. Therefore, bacteriocins from lactic acid bacteria (LAB) are 'natural' inhibitors that appear highly attractive (3).

Bacteriocins inhibit spoilage and pathogenic bacteria without changing (through acidification, protein denaturing, etc.) the physico-chemical nature of the food being preserved. Another contributory factor for the increased interest in bacteriocins is that their proteinaceous nature implies their degradation in the gastrointestinal tracts of man and animals.

LAB have been studied extensively for bacteriocinogenicity, and numerous bacteriocins are produced by LAB (4, 5). Lactobacilli that produce bacteriocins have been cultured from naturally fermented dairy products, non-dairy fermentations (plant and meat), starter cultures, and plant, animal, or human isolates. Among the numbers of LAB, the lactobacilli are composed of a diverse group of homofermentative and heterofermentative species. They are most often cited for production of bacteriocins (4). Although lactobacillus bacteriocins are generally active against closely related types which occupy similar ecological niches, some are broader-acting bacteriocins such as sakacin A (6) and lactacin F (7).

Recently we identified a bacteriocin-producing lactobacilli, *Lactobacillus buchneri* LB, and its bacteriocin was designated buchnericin LB (8). The objectives of the present study were to determine its inhibitory effects against spoilage and pathogenic microorganisms, and the physico-chemical characteristics and stability of buchnericin LB.

Materials and Methods

Strains and culture conditions

Lactobacillus buchneri LB tested for antimicrobial activity were isolated from a commercially fermented vegetable product, sauerkraut. For identification of the purified strain, the general microbiological tests, carbohydrate fermentation (Strep API-20, API-50 CHL and BioLog) and fatty acid profile (MIDI) identification systems were used. The strain was gram-positive and catalase-negative, and hydrolyzed esculine and arginine, but not gelatin and starch. It did not produce α- and β-hemolysis on blood agar, and grew between 15-40°C and at pH 4.5-9.2. In addition, it fermented melezitose, arabinose, maltose, ribose and melibiose, whereas it did not ferment mannitol, mannose and cellobiose. The major fatty acids found in the isolate were 16:0 (25.55%) and 18:1 (37.33%). Based on these results, it was identified as *Lb. buchneri* LB (8). *Lb. buchneri* LB was maintained in frozen stock cultures at -70°C in de Mann Rogosa Sharpe (MRS) broth containing 20% glycerol. Lactic acid and other bacteria (indicator organisms) were grown in MRS broth and BHI broth, respectively (Table).

Preparation of crude buchnericin LB

Lb. buchneri LB grown in MRS broth (1500 mL) at 30°C for 18 h was divided into 50 mL fractions. These samples were adjusted to pH levels ranging from 2.0 to 9.0 with combinations of 5 mol L^{-1} NaOH and 5 mol L^{-1} phosphoric acid. The samples were heated to 80°C for 25 min to kill the cells and mixed with a magnetic stirrer overnight at 4°C for adsorption of buchner-

Table. Antimicrobial spectrum of buchnericin LB

Bacteria & Source	Antimicrobial Activity[a]	Diameter of inhibition zone (mm)
Listeria ivanovii ATCC 19119	+	24±0.08
Listeria innocua ATCC 25401	+	25±0.05
Listeria grayi ATCC 19120	+	23±0.06
Listeria monocytogenes Scott A USDA	+	21±0.09
Listeria monocytogenes ATCC 19113	+	22±0.07
Enterococcus faecalis ATCC 6055	+	18±0.10
Enterococcus faecium ATCC 6057	+	19±0.12
Bacillus cereus ATCC 232	+	14±0.06
Bacillus subtilis ATCC 15244	+	10±0.08
Lactobacillus viridans UMRL	+	18±0.10
Lactobacillus acidophilus ATCC 9224	+	20±0.04
Lactobacillus plantarum NCDO 955	+	27±0.08
Lactobacillus casei ATCC 334	+	24±0.11
Leuconostoc oenos UMRL	+	14±0.05
Leuconostoc mesenteroides NCDO 529	+	13±0.06
Pediococcus acidolactici ATCC 25440	+	18±0.12
Pediococcus dextrinicus ATCC 19371	+	16±0.14
Pediococcus urinaqui ATCC 29723	+	19±0.10
Micrococcus roseus ATCC 14396	+	18±0.08
Micrococcus luteus ATCC 4698	+	14±0.06
Streptococcus thermophilus NCDO 573	+	11±0.05
Staphylococcus aureus ATCC 25923	-	NA[b]
Clostridium perfringens ATCC 12924	-	NA
Clostridium sporogenes ATCC 17887	-	NA
Lactococcus lactis ATCC 11454	-	NA
Corynebacterium glutamicum ATCC 31834	-	NA
Shigella flexneri ATCC 12022	-	NA
E. coli 0157:H7 ATCC 43895	-	NA
Salmonella typhimurium ATCC 14028	-	NA

[a]+, inhibition; -, no inhibition; [b]NA, not applicable

icin LB onto the producer cells. For desorption of the bound bacteriocin from the cells, cells were washed with 5 mol L^{-1} sodium phosphate (pH 6.5), resuspended in 50 mL of 100 m mol L^{-1} NaCl at pH 2.0 (adjusted with 5% phosphoric acid) and stirred at 4°C for 2h. After centrifugation at 1000 x g for 30 min, the supernatants containing free buchnericin LB were filter sterilized (0.22 µm pore size, Millipore), dialyzed against deionized water in a 1000-molecular weight-cutoff dialysis bag at 4°C for 24 h, and freeze dried (9, 10, 11). This final product (15 ml) was termed crude bacteriocin.

Determination of molecular weight of buchnericin LB

Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE) was performed according to the method of Bhunia et al. (12) and Schagger and von Jagov (13) in 10-20% tricine-SDS-PAGE gradient gel. After electrophoresis, the gel was sliced into two pieces. One half of the gel was stained with Coomassie Blue G-250 for 1 h and destained until the background stain disappeared. For antimicrobial assay, the other half was stained for 30 min and then destained for 1.5 h. After the gel was equilibrated in changing deionized water for 3 h to remove destained solution (acetic acid and methanol), it was placed carefully on the surface of MRS agar plate. The gel was seeded with 30 ml of tempered an MRS soft agar containing *Lb. plantarum* NCDO 955 as an indicator organism and incubated at 30°C for 24 h.

Detection of antimicrobial activity

For detection of antimicrobial activity against food spoilage bacteria and foodborne pathogens as indicator organisms, the agar spot test was used (14). For the agar spot test, 10 µl of the crude bacteriocin preparation was spotted onto the surface of agar plates (MRS or BHI) which had been overlaid with indicator organisms in 5 ml of soft MRS or BHI agar. These plates were incubated at 30°C or 37°C depending on the indicator strains. After incubation for 24 h, the plates were checked for inhibitory zones. Inhibition was scored positive if the zone was wider than 2 mm.

To determine the arbitrary unit (AU/mL) of the bacteriocin, the most sensitive indicator, *Lb. plantarum* NCDO 955, was chosen to assay for buchnericin LB activity. Titrations were performed by the serial dilutions assay described by Yıldırım and Johnson (10, 11) and activity was assigned as arbitrary units per milliliters. The arbitrary unit (AU/mL) was calculated as the reciprocal of the highest dilution, in 10 µL, which gave a clear zone of growth inhibition on a lawn of sensitive cells (10, 11, 15).

Sensitivity to heat and enzymes

Thermostability of the antimicrobial activity was determined by heating 2 mL (10 mg mL^{-1}) samples of the crude buchnericin LB preparations at 90°C for 15, 30 or 60 min or at 121°C for 15 min; they were then cooled and tested for residual activity. To determine stability to storage conditions, buchnericin preparation (10 mg mL^{-1}) was kept at 4°, -20° or -70°C for 3 months. The sensitivity of buchnericin LB to enzymes was assayed by incubating cell-free super-

natant fluids with 1 mg/mL pepsin, trypsin, proteinase K, α-chymotrypsin, pronase E, papain, catalase, peroxidase, lipase, lysozyme, α- and β-amylases (Sigma) in 4 mL mol L^{-1} phosphate buffer, pH 7.0, at 37°C for 1 h. The residual activity of the samples was determined by the agar spot test (14).

Stability of buchnericin LB at different pH values and in various solvent solutions

Crude bacteriocin preparations (50 mg mL^{-1}) were adjusted to various pH values (2.0-12.0) with 5 mol L^{-1} NaOH or 5 mol L^{-1} HCl and the final concentration was brought to yield 10 mg/mL. These samples were incubated at 25°C for 2 or 24 h, or at 90°C for 20 min, readjusted to pH 6.0, and then assayed for residual inhibitory activity (10, 11).

Crude buchnericin LB was dissolved in various organic solvents – formaldehyde, chloroform, acetone, 2-propanol, ethyl alcohol, hexane, iso-butanol and ethyl ether – at a concentration of 10 mg mL^{-1}. After incubation at 25°C for 1 h, solvents were evaporated in a centrifugal concentrator. Dried samples were reconstituted with sterile deionized water to a concentration of 10 mg mL^{-1} and assayed for antimicrobial activity (10, 11, 16).

Result

Buchnericin LB preparation, molecular weight and inhibitory spectrum

Antimicrobial activities of crude buchnericin LB in the cell-free supernatants (1.5 L) and the supernatant (15 mL) obtained after adsorption/desorption from heat-killed producer cells were 3,200 AU/mL (4,800,000 AU) and 128,000 AU/mL (1,920,000 AU), respectively. Forty percent of buchnericin LB bound to heat-killed cells was desorbed at pH 2.0 by using 100 mol L^{-1} NaCl. It was found that 95% of buchnericin LB adsorbed to the heat-killed cells at pH 5.0-7.0 whereas at pH values below 5.0 or above 7.0, its adsorption decreased (Fig. 1).

Tricine-SDS-PAGE gel stained with Coomassie Blue revealed more than three bands from the buchnericin LB preparation (Fig. 2). The gel overlaid with *Lb. plantarum* showed a clear zone of inhibition over the 3.8 kDa protein band (Fig. 2).

Buchnericin LB had inhibitory activity against several foodborne pathogens and food spoilage bacteria including *Listeria, Bacillus, Enterococcus, Lactobacillus, Leuconostocs, Micrococcus, Pediococcus* and *Streptococcus* species (Table).

Heat, enzyme and organic solvent sensitivity of buchnericin LB

The inhibitory activity of buchnericin LB preparation showed stability to heat. The activity of buchnericin LB was decreased by 25 and 50%, only after heating at 90°C for 30 and 60 min respectively, and by 25% after autoclaving at 121°C for 15 min. The initial activity of buchnericin LB did not decrease when stored at -20 or -70°C for 3 months whereas the activity of samples stored at 4°C for 1 or 3 months decreased by 25 or 50%, respectively (Fig. 3).

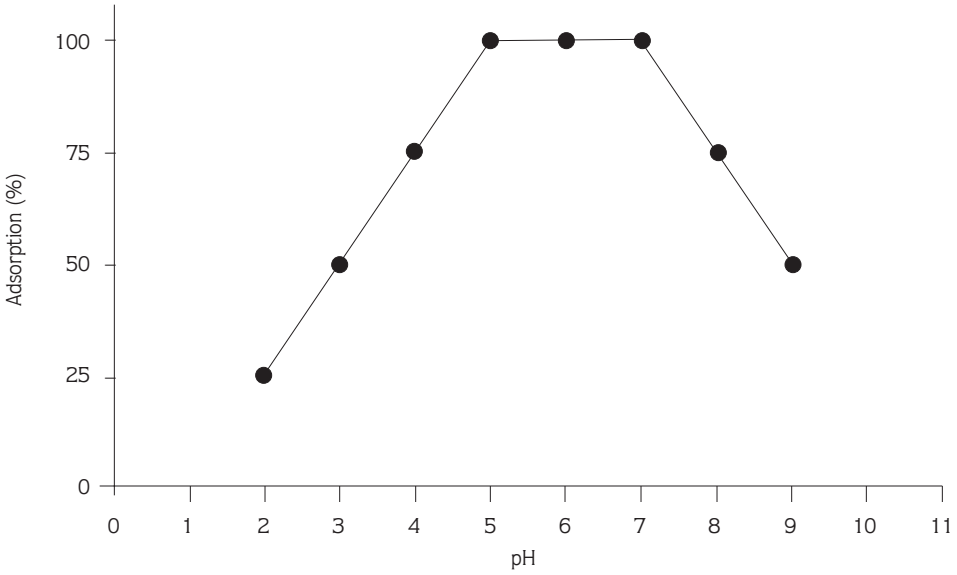

Fig. 1. Effect of pH on adsorption of buchnericin LB to heat-killed *Lactobacillus buchneri* LB cells.

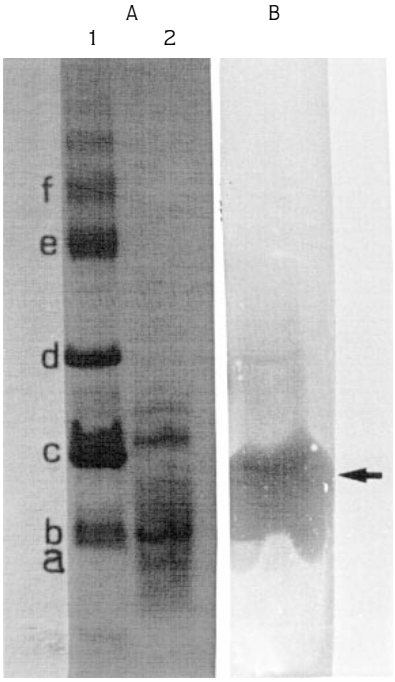

Fig.2. A, tricine-SDS-PAGE gel electrophorosis of buchnericin LB preparation. Lane 1, low molecular weight standards (Bio-Rad): a, 1.423; b, 3.496; c, 6.512; d, 14.437; e, 16.950; f, 26.625 kDa. Lane 2, buchnericin LB preparation; B, gel overlay with *Lb. plantarum* to determine which band corresponds to the antimicrobial activity and molecular weight.

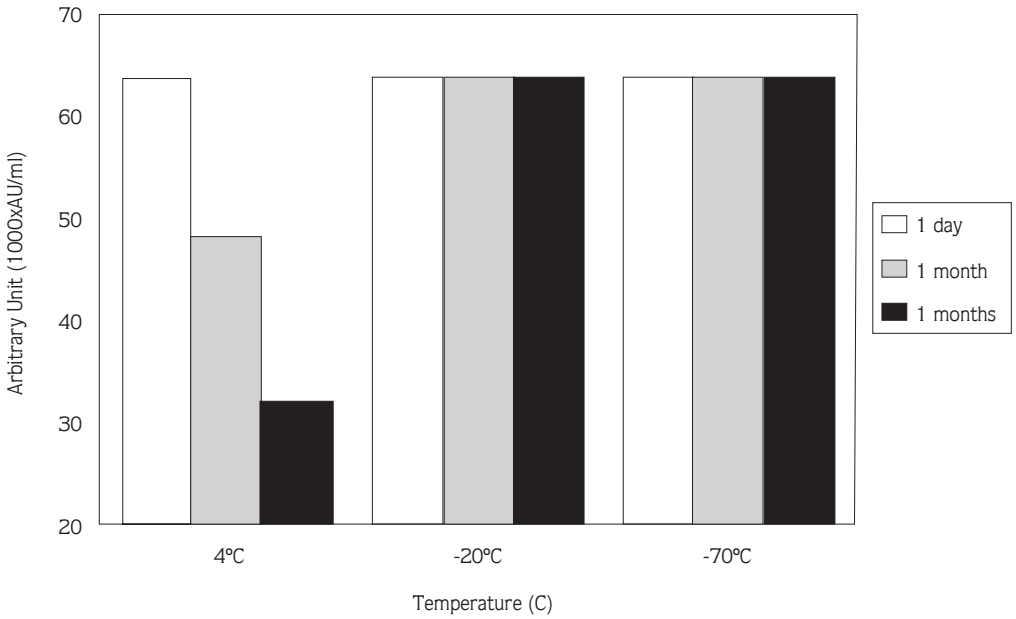

Fig. 3. Effect of storage temperature on inhibitory activity of buchnericin LB.

Buchnericin LB lost its activity completely after treatment with certain proteases, α-chymotrypsin, trypsin, proteinase K, pepsin and pronase E; however, it was resistant to non-proteolytic enzymes such as lysozyme, ribonuclease A, catalase, peroxidase, amylases and lipase. In addition, it retained its biological activity after treatment with the organic solvents tested.

Effect of pH on buchnericin LB activity

The pH of culture supernatant fluids from *Lb. buchneri* LB at the end of logarithmic growth phase was adjusted to a range of values (pH 2.0-9.0). The highest bacteriocin titer of inhibitory effect on *Lb. plantarum* NCDO 955 was found at pH 4.0-7.0 (3,200 AU/mL). To determine bacteriocin stability, crude buchnericin LB preparations were adjusted to different pH values (2.0-12.0) and incubated at 25°C for 2 or 24 h, or at 90°C for 30 min, and then pH was readjusted to 6.0. Full activity was retained when incubation was carried out at pH 4.0-7.0 under all three conditions (Fig. 4). The activity of buchnericin LB was decreased by 25-50% at pH 3.0 or below and by 25-75% at pH 8.0 or above. Under all conditions, buchnericin LB did not show inhibitory activity at pH 11.0 and 12.0.

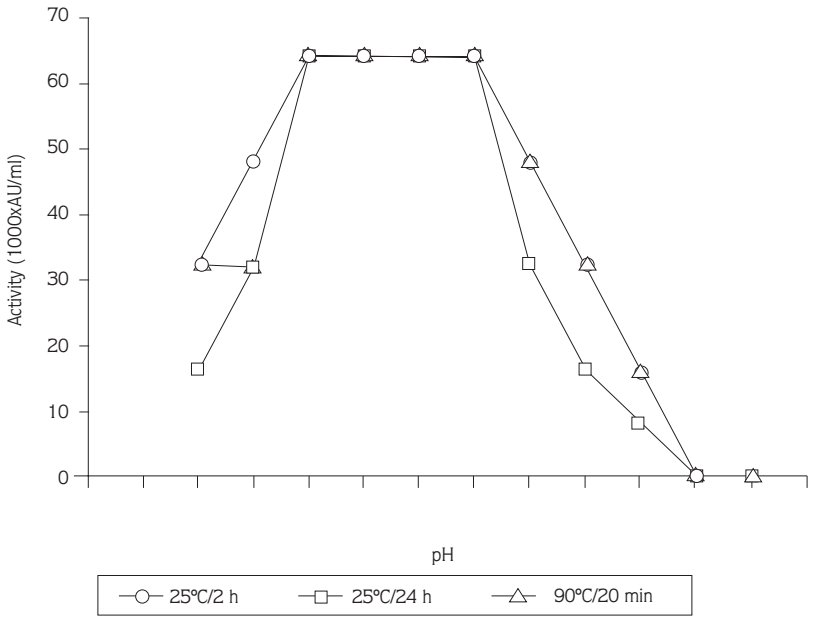

Fig. 4. Effect of pH on the inhibitory activity of buchnericin LB

Discussion

In this study, the physicochemical and antimicrobial spectra, molecular weight and stability of buchnericin LB produced by *Lb. buchneri* were determined.

Buchnericin LB is a low-molecular-weight antimicrobial peptide. Its molecular size is about 3.5-4.5 kDa according to SDS-PAGE. Buchnericin LB has a wide inhibitory spectrum since it is active against more than three genera. It was found to be active towards lactic acid bacteria such as the *Lactobacillus, Leuconostoc, Enterococcus, Pediococcus* and *Streptococcus* species tested. In addition, strains of spore-forming microorganisms such as *Bacillus* and foodborne pathogenic bacteria such as *Listeria monocytogenes* were inhibited by buchnericin LB. These results show that sensitivity was species-specific rather than strain-specific. A broad spectrum of activity has also been described for other lactobacillus bacteriocins (17, 18). Buchnericin LB did not show antimicrobial activity against some lactic acid bacteria and gram-negative bacteria. This shows that these bacteria do not have specific receptors for buchnericin LB.

Like other most lactic acid bacteria, buchnericin LB appeared to aggregate and precipitate with other proteins during dialysis against deionized water (10, 11, 16, 19). These aggregates had antimicrobial activity. Adsorption of buchnericin LB onto heat-killed producer cells was strongly affected by the pH of the suspending environment. The maximum adsorption occurred at pH 5.0-7.0.

Buchnericin LB completely lost its biological activity after treatment with proteases, trypsin, α-chymotrypsin, pepsin, papain and pronase E. These results indicate that the inhibitory agent is a protein since protease sensitivity is a key criterion for the characterization of a bacteriocin. After treatment with lipase, amylase or organic solvents, buchnericin LB did not lose its inhibitory activity. These results show that lipid and carbohydrate moieties were not responsible for its antimicrobial activity. In addition, inhibitory action against sensitive organisms was not due to hydrogen peroxide or acidity because treatment with catalase or peroxidase or adjustmet of pH to 7.0 did not cause any apparent loss of buchnericin LB activity.

Buchnericin LB retained its antimicrobial activity after exposure to pH 2.0-9.0. It was relatively more stable to high temperatures in acidic conditions than in basic conditions. It was also resistant to high temperatures and long storage periods.

Since the first description of bacteriocins produced by lactobacilli, more than 25 different bacteriocins have now been reported (4). Most bacteriocins produced by *Lactobacillus* are generally active against closely related types which occupy similar ecological niches. Buchnericin LB has a wide inhibitory spectrum, inhibiting the growth of *Listeria, Bacillus, Enterococcus, Micrococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* species. This is the first study in which *Lb. buchneri* is found to produce a bacteriocin.

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