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Factors affecting the adsorption of buchnericin LB, a bacteriocin produced by *Lactocobacillus buchneri*

Zeliha Yıldırım[1], Yahya Kemal Avşar[2], Metin Yıldırım[1]

[1] Gaziosmanpaşa University, Faculty of Agriculture, Department of Food Engineering, Tokat, Turkey
[2] Mustafa Kemal University, Faculty of Agriculture, Department of Dairy Technology, Antakya, Turkey

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Abstract

Buchnericin-LB adsorbs to gram-positive but not to gram-negative bacteria. The tested gram-positive bacteria were species of *Lactobacillus, Pediococcus, Leuconostoc, Enterococcus, Lactococcus, Listeria, Bacillus, Staphylococcus;* gram-negative bacteria belonged to the genera *Salmonella, Escherichia, Yersinia* and *Pseudomonas.* Buchnericin-LB adsorption depended on pH but not on time and temperature. Also some anions of salts and lipoteichoic acid reduced or inhibited its adsorption. Treatment of cells and cell walls of sensitive bacteria with detergents, organic solvents or enzymes did not affect subsequent binding of buchnericin-LB. Treatment with buchnericin-LB caused sensitive cells to lose high amounts of intracellular K^+ ions and UV-absorbing materials and became more permeable to o-nitrophenol-β-D-galactopyranoside. Buchnericin-LB (640-2560 AU/ml) decreased the colony forming units (99%) and absorbance values of *Listeria monocytogenes* and *Bacillus cereus*. These results indicate that the mode of action of buchnericin-LB is bactericidal and its lethal effect is very rapid.

Key words: Buchnericin LB – adsorption – bacteriocin – *Lactobacillus buchneri* – lipoteichoic acid

Introduction

Natural antimicrobial compounds present in food such as lactoperoxidase or lysozyme, endogenous milk proteins such as lactoferrin and lactoferricin, peptides produced by bacteria such as nisin and pediocins, are being explored for improving the safety of a wide variety of processed foods.

Corresponding author: Z. Yildirim
e-mail: yildirimzeliha@hotmail.com

Bacteriocins are proteinaceous compounds produced by bacteria that exhibit a bactericidal or bacteriostatic mode of action against sensitive bacterial species (Tagg *et al.* 1976; Klaenhammer 1988; Klaenhammer 1993). Bacteriocins have gained increasing interest since these substances exert a broad spectrum of inhibitory activity among bacteria. Therefore, antimicrobial proteins produced by lactic acid bacteria are excellent candidates to improve the safety of various fermented foods (Barefoot and Klaenhammer 1984; Piard *et al.* 1992).

Yildirim and Yildirim (2001) have described a bacteriocin, termed buchnericin LB produced by *Lactobacillus buchneri*. Buchnericin LB inhibited the growth of some species of *Listeria, Bacillus, Micrococcus, Enterococcus, Leuconostoc, Lactobacillus, Streptococcus* and *Pediococcus*. Its molecular weight ranges between 3.5 and 4.5 kDa. Buchnericin LB is sensitive to proteolytic enzymes but resistant to a broad pH between 2.0 and 9.0, heat up to 90–121 °C for 15 min and organic solvents such as formaldehyde, chloroform, hexane, 2-propanol, ethanol, iso-butanol, and ethyl ether at concentrations of 10 mg/ml.

In order to use buchnericin LB in the most effective way, it is important to determine its mode of action and factors affecting its adsorption to indicator organisms. The aims of this study were to determine the mode of action of buchnericin LB and factors affecting its adsorption to sensitive bacteria.

Materials and methods

Bacterial culture and preparation of crude buchnericin LB. Lactobacillus buchneri was grown in de Mann Rogosa Sharpe (MRS) broth. To prepare buchnericin

LB, *L. buchneri* was inoculated into MRS broth (1.5 l) and incubated at 30 ± 2 °C. After incubation, cell free-supernatant was collected by centrifugation (1,000 × g for 20 min), passed through sterile filters (0.45 µm pore size), freeze dried and reconstituted to 150 ml with sterile distilled water. These samples were partially purified using silicic acid (Coventry *et al.* 1996; Yildirim 2001).

Cell wall preparation. Cell walls of *Lactobacillus plantarum* grown in 200 ml of MRS broth at 30 °C for 20 h were prepared according to published procedures (Sprott *et al*. 1996; Yildirim *et al.* 1999).

Adsorption of buchnericin LB to indicator organisms. Adsorption of buchnericin LB to indicator cells, gram-positive and gram-negative bacteria, was done according to Bhunia *et al.* (1991). After indicator organisms were grown overnight in MRS or BHI broth at 30 or 37 °C, they were centrifuged at 7,000 g for 15 min. Cells were washed twice with sterile 5 mM phosphate buffer (pH 6.6) and resuspended to the original volume in the same buffer. Each cell suspension (1 ml) was mixed with buchnericin LB (3,200 AU/ml) and incubated at 25 °C for 30 min. After removal of cells by centrifugation, unbound buchnericin LB activities in the supernatant fluids and cells were determined.

Effect of temperature, pH, time, lipoteichoic acid and various chemicals on adsorption of buchnericin LB to Lactobacillus plantarum. The effect of temperature (0, 10, 25, 50 or 85 °C), pH (2, 3, 4, 5, 6, 7, 8, or 9), time (0.5, 5, 10, 20, 30 or 60 min), lipoteichoic acid from *Enterococcus faecalis* (Sigma) (0.1 ml of 10 mg/ml in distilled water), and different salts or protein solutions on adsorption of buchnericin LB were determined using the method described in previous studies (Yildirim *et al*. 1999; Bhunia *et al.* 1991).

Effect of detergents, solvents and enzymes on adsorption of buchnericin LB to whole cells and cell walls of Lactobacillus plantarum. The method of Bhunia *et al.* (1991) was used to determine the effects of detergents, solvents and enzymes on adsorption of buchnericin LB.

Effect of buchnericin LB on the growth of Listeria monocytogenes and Bacillus cereus. Various concentrations of buchnericin LB (0, 640, 1,280 or 2,560 AU/ml) were added to each sample of actively growing *L. monocytogenes* and *B. cereus* cells in Brain Hearth Infusion (BHI) broth. After incubation at 37 °C for 4 h, samples were periodically withdrawn to determine the cell viability and sample absorbance at 600 nm.

Effect of buchnericin LB treatment on the release of cytoplasmic materials in the environment. Buchnericin LB (640 AU/ml) was added *L. plantarum* cells suspended in 5 ml of phosphate buffer (5 mM, pH 5.5) and control samples were cells without bacteriocins and buffer with buchnericin LB but no cells. After incubation at 37 °C for 45 min, the samples were centrifuged, filtered through 0.22 µm pore size sterile membranes (Millipore) and read at 260 nm by using a spectrophotometer. The concentration of potassium ions in the filtrates was measured by atomic adsorption spectroscopy (Yildirim *et al.* 1999).

Measurement of o-nitrophenol-β-D-galactopyranoside (ONPG) permeability on Lactobacillus plantarum cells treated with buchnericin LB. After buchnericin LB (640 AU/ml) was added to *L. plantarum* cells (2 ml) in phosphate buffer (5mM, pH 5.5), it was incubated at 25 °C for 5 min and then 0.1 ml of o-nitrophenol-β-D-galactopyranoside (ONPG); (0.1 M) was added to each sample and incubated at 37 °C for 10 min. The absorbances of the cell free supernatant collected by centrifugation (7,000 g for 15 min) were measured at 420 nm in a Beckman DU-50 spectrophotometer. Controls were either *L. plantarum* cells or buchnericin LB in the same buffer (Bhowmik *et al.* 1987).

Results

Adsorption of buchnericin LB to indicator organisms

Buchnericin LB adsorbed to both sensitive and resistant cells of gram-positive bacteria tested, but it did not adsorb to the gram-negative bacteria tested (Table 1). Adsorption ranged from 80–100%.

Effect of various physical and chemical factors on adsorption of buchnericin LB

It was found that the adsorption of buchnericin LB was maximal between pH 5.0 and 8.0 (100%), but below or above these values the adsorption was decreased to 50%. The adsorption of buchnericin LB (100%) was identical for all temperatures tested (0, 10, 25, 50, 80 °C).

To study the effect of time on binding of buchnericin LB, *L. plantarum* had been exposed to buchnericin LB for 0.5, 5, 10, 20, 30 or 60 min. *L. plantarum* did not grow during the 4 h of the experiment (Fig. 1). However, the control cells under the same condition grew very well. In addition, 95% of buchnericin LB was adsorbed within 0.5, 5, or 10 min, and adsorption reached 100% after 20 min.

The binding of buchnericin LB to *L. plantarum* cells was completely prevented by addition of purified lipoteichoic acid from *E. faecalis* (Table 2). NaCl, sodium phosphate, NH_4Cl, $MgCl_2$, KCl, KI or Tris-HCl resulted in a reduction in adsorption of buchnericin LB to indicator organism, whereas NH_4-citrate, Na-acetate,

Table 1. Adsorption of buchnericin LB to resistant and sensitive gram-positive and gram-negative bacterial species

Sensitive organisms	Adsorption (%)	Resistant organisms (%)	Adsorption
Lactobacillus plantarum (Gr +)	100	*Lactococcus lactis* (Gr +)	94
Pediococcus dexrinicus (Gr +)	100	*Pediococcus cerevisiae*(Gr +)	100
Leuconostoc oenos (Gr +)	100	*Staphylococcus aureus* (Gr +)	80
Enterococcus faecalis (Gr +)	100	*Salmonella typhimurium*(Gr –)	0
Listeria monocytogenes (Gr +)	95	*Escherichia coli* (Gr –)	0
Listeria ivanovii (Gr +)	90	*Yersinia enterocolitica* (Gr –)	0
Bacillus cereus (Gr +)	95	*Pseudomonas fluorescens* (Gr –)	0

Gr + : Gram positive
Gr – : Gram negative

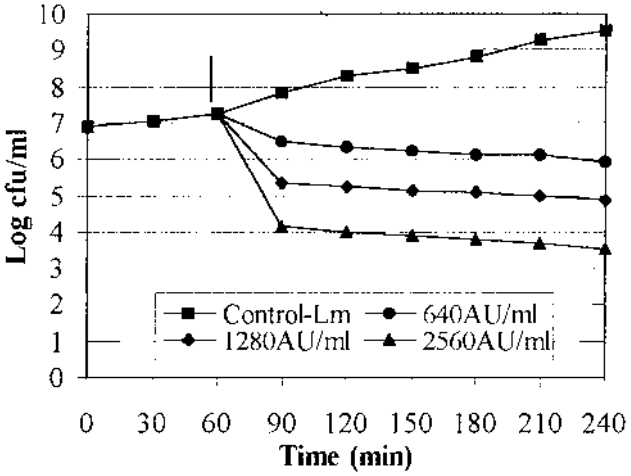

Fig. 1. Effect of buchnericin LB on growth of *Listeria monocytogenes* in BHI broth at 37 °C. Bacteriocin was added at 60 min.

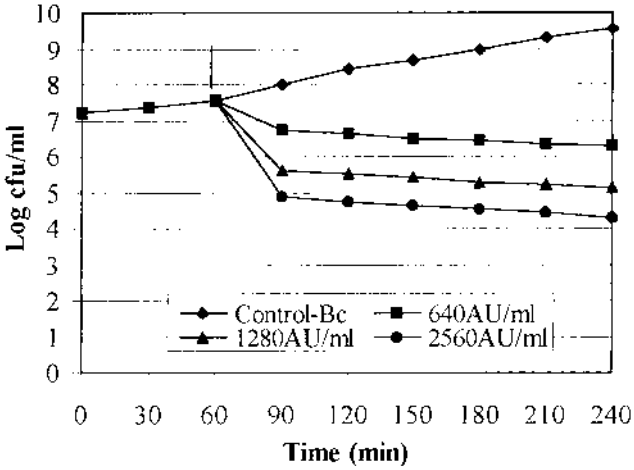

Fig. 2. Effect of buchnericin LB on growth of *Bacillus cereus* in BHI broth at 37 °C. Bacteriocin was added at 60 min.

$NaCO_3$, EDTA, bovine serum albumin or casein had no effect on adsorption (Table 2).

Whole cells and cell walls of *L. plantarum* treated with 4M guanidine-HCl, 1% SDS, 2% triton-X, organic solvents such as 0.5% 2-mercaptoethanol, 80% ethanol, 80% hexane, 80% acetone and 80% methanol, and enzymes (protease, trypsin, lipase, lysozyme) had no effect on adsorption of buchnericin LB. Treatment of cell walls with a mixture of methanol and chloroform (40 : 40, v/v) followed by hot 20% TCA prevented the binding of buchnericin LB. Methanol:chloroform (40 : 40, v/v) plus cold 20% TCA treatment decreased adsorption by 30%.

Effect of buchnericin LB on the growth of Listeria monocytogenes and Bacillus cereus

Addition of buchnericin LB at different levels (0–2,560 AU/ml) caused a decrease in the colony counts and absorbance values (600 nm) of growing cultures of *L. monocytogenes* and *B. cereus* in BHI broth in proportion to the amount of buchnericin LB added (Fig. 1 and 2). After addition of buchnericin LB, the colony counts decreased by 0.7, 1.92 and 3.12 log for *L. monocytogenes* and 0.8, 1.92 and 2.86 for *B. cereus* at the levels of 640, 1,280 and 2,560 AU/ml within 30 min, respectively. After 4 h incubation, the colony forming units declined by 1.34, 2.39 and 3.75 log for *L. monocytogenes* and 1.28, 2.44 and 3.24 for *B. cereus* at the respective buchnericin LB levels.

Effect of buchnericin LB on the release of cellular materials

L. plantarum treated with pure buchnericin LB resulted in leakage of UV-absorbing materials and potassium ions. In these samples, an increase in 260 nm absorbing materials compared with control was observed (Table 3). Potassium ion efflux and UV-absorbing materials in-

Table 2. Effect of the presence of lipoteichoic acid and various chemicals on adsorption of buchnericin LB to *Lactobacillus plantarum*.

Treatments	Adsorption (%)
Control (cells+lactococcin R)	100
Lipoteichoic acid (1 mg/0.1 ml)	0
NaCl (0.3 M)	75
Na-phosphate (0.3 M)	85
NH4-citrate (0.1%)	100
NH4Cl (0.3 M, pH 5.5)	95
MgCl2 (0.3 M, pH 6.5)	80
KCl (0.3 M, pH 5.6)	90
KI (0.3 M, pH 6.6)	85
Na-acetate (0.5%)	100
NaCO3 (0.3 M, pH 7.6)	95
EDTA (0.01 M)	100
Tris-HCl (0.6 M, pH 6.8)	50
Tris-HCl (0.02 M, pH 6.8)	90
Bovine serum albumin (0.5%)	100
Casein (0.5%)	100

Table 3. Effect of buchnericin LB on the release of cellular materials from *Lactobacillus plantarum* cells

Parameters	Untreated cell supernatant	Buchnericin LB solution	Treated cells supernatant fluid
Absorbance (260 nm)	0.160	0.139	2.19
Potassium ions (mg/l)	37	2.65	479
ONP (mM)	192	0	1569

creased 11.5 and 13.5 fold in buchnericin LB treated cells, respectively. In addition, treated cells became 10 times more permeable to ONPG than the untreated cells (Table 3).

Discussion

In this study, the possible mode of action of buchnericin LB and factors affecting its adsorption were investigated. It was observed that buchnericin LB adsorbed to all gram-positive bacteria; it did not adsorb to the gram-negative bacteria tested. Gram-negative bacteria probably do not have receptors for the adsorption of buchnericin LB (Bhowmik *et al.* 1987; Upreti and Hinsdill 1975; Wicken and Knox 1975). Adsorption of buchnericin LB resulted in cell death in sensitive gram-positive cells, but not in the resistant gram-positive bacteria. These results indicate that sensitive bacteria have both, specific and non-specific receptors, whereas resistant bacteria have only non-specific receptors. In sensitive cells, adsorption and lethal action of buchnericin LB was very rapid since in 0.5 min it adsorbed to *L. plantarum* cells by 95% and inhibited its growth. Also, it was found that time, low and high temperatures did not affect the binding of buchnericin LB to indicator organisms.

The presence of salts such as sodium chloride, sodium phosphate, ammonium chloride, magnesium chloride and potassium chloride reduced the adsorption of buchnericin LB. These results show that ionic interactions may involve the adsorption of buchnericin LB to cell surface receptors. The addition of purified lipoteichoic acid to cells blocked the binding of buchnericin LB to indicator organisms. Buchnericin LB probably forms a complex with lipoteichoic acid making it unavailable for adsorption to the target cells. Therefore, it is unable to inhibit cell growth (Yildirim *et al.* 1999; Bhunia *et al.* 1991). This explains why buchnericin LB is adsorbed to the gram-positive bacteria, but not to the gram-negative bacteria. Lipoteichoic acid molecules probably are the non-specific receptors and thus are associated with binding of buchnericin LB in both, resistant and sensitive gram-positive bacteria (Yildirim *et al.* 1999; Bhunia *et al*. 1991; Upreti and Hinsdill 1975).

Treatment of cell or cell walls of *L. plantarum* with different enzymes, several organic solvents or detergents had no effect on adsorption of buchnericin LB. These results show that the binding sites of buchnericin LB are probably not proteins, carbohydrates or lipids. However, cell walls treated with methanol : chloroform plus hot 20% TCA lost their ability of binding of buchnericin LB. This result also demostrates a possible involvement of cell wall lipoteichoic acid in the binding of buchnericin LB (Yildirim *et al.* 1999; Pucci *et al.* 1988).

The lethal action of buchnericin LB on sensitive cells caused the loss of UV-absorbing materials and potassium, associated with membrane damage. Similar results were reported earlier (Yildirim *et al.* 1999; Bhunia *et al.* 1991; Wicken and Knox 1975).

The mode of action of buchnericin LB is bactericidal to sensitive bacterial cells rather than bacteriostatic. Cell death appears to be associated with lysis of the cell membrane or subsequent cytosol leakage.

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