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Antibacterial Activity of *Lactobacillus buchneri* Bacteriocin against *Vibrio parahaemolyticus*

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Abstract

Eleven yoghurt samples were collected from local markets in Baghdad to isolate *Lactobacillus buchneri*. Only 3 isolates of *L. buchneri* were found and the isolate No. 3 was the most producer of bacteriocin. Bacteriocin was adsorbed 100% onto silicic acid at pH 6.0-7.0. Below or above these pH values, adsorption was decreased, ranging between 35 and 90%. Therefore, pH 6.0 was used for the purification procedure. The purification procedure including silicic acid adsorption/desorption and cation-exchange chromatography (CEC) resulted in a 11.11 fold increase in the final specific activity of pure bacteriocin (1176.47 Au/mg) compared to the culture supernatant which was 32.64 Au/mg. The molecular weight was determined to be about 3.4 kDa. The bacteriocin lost its activity completely after treatment with proteolytic enzymes and it was resistant to non-proteolytic enzymes. The results indicated that bacteriocin at both concentrations (500 and 1000 μg/ml) possesses significant antimicrobial activity against *Vibrio parahaemolyticus* in contrast with control ($P<0.01$) and the antimicrobial activity of crude and purified bacteriocin at the concentration of 1,000 μg/ml were higher than the other concentration (500 μg/ml). The antimicrobial activity of purified bacteriocin was significantly higher than that of crude bacteriocin ($P<0.01$).

Keywords: antimicrobial activity, Buchnericin LB, *Lactobacillus buchneri*

1. Introduction

Lactic acid bacteria (LAB) have been studied extensively for bacteriocinogenicity, and numerous bacteriocins are produced by LAB [1, 2]. 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 compose of a diverse group of homofermentative and heterofermentative species. They are most

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often cited for production of bacteriocins [1]. Bacteriocins are antimicrobial proteinaceous compounds produced by bacteria [1]. *Lactobacillus buchneri* is a heterofermentative, facultative anaerobe that belongs to the lactic acid 2 bacteria. Strains of *L. buchneri* have been described as having diverse activities, ranging from prevention of silage spoilage by yeasts and molds [3-5] to histamine production in Swiss cheese [6]. Yildirim and Yildirim [7] have described a bacteriocin, termed buchnericin LB produced by *L. buchneri*. Buchnericin LB inhibited the growth of some species of *Listeria, Bacillus, Micrococcus, Enterococcus, Leuconostoc, Lactobacillus, Streptococcus* and *Pediococcus*. In order to use buchnericin LB in the most effective way, it is important to determine factors affecting its adsorption to indicator organisms. The aims of this study were to determine factors affecting buchnericin LB adsorption to indicator bacteria, *Vibrio parahaemolyticus*.

2. Materials and Methods

2.1 Sample collection

Eleven yoghurt samples were collected from local markets in Baghdad to isolate *L. buchneri*. The isolates were incubated in lactobacilli MRS broth as growth medium at 37 ºC for 24 h.

2.2 Identification of *Lactobacillus buchneri*

The properties of the isolates were investigated by Gram staining and microscopic observation after cultivation on tryptic soy agar medium at 37 ºC for 24 h. Bergey's Manual of Systematic Bacteriology was used to examine the morphological and physiological properties of the isolates [8]. The API 50 CHL-kit (Bio-Merieux, France), an identification system for LAB was used to identify *L. buchneri* isolates.

2.3 *Vibrio parahaemolyticus* isolates

Six isolates of *V. parahaemolyticus* isolated from clinical sources were used in this study. All isolates were confirmed with API 20E (Bio-Merieux, France). This system was used according to Bio-Merieux company instruction.

2.4 Production of crude bacteriocin

MRS broth (Hi Media Laboratory Pvt. Ltd. India) at pH 6.0 was seeded with 5% inoculum of *L. buchneri* overnight culture and maintained anaerobically at 30 ºC for 48 h. After incubation, cells were removed from the growth medium by centrifugation (10,000xg for 15 min. at 4 ºC). The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin [9].

2.5 Purification of the bacteriocin

For adsorption of bacteriocin onto silicic acid (100 mesh), freeze-dried supernatants reconstituted with 150 ml of distilled water and fractionated into 10 ml. Bacteriocin preparations in 10-ml fractions were adjusted to pH 2.0-9.0 with 5 M phosphoric acid or 5 M NaOH, and their volumes were brought up to 15 ml with distilled water. After silicic acid (2%) purification the bacteriocin produced by *L. buchneri* LB was added into each sample and stirred overnight at 4 ºC before centrifugation at 1,000 x g for 20 min. Bacteriocin adsorbed silicic acid was washed with sterile distilled water and re-suspended to the original volume of 15 ml with 100 mM NaCl. In order to desorb the bound bacteriocin, the pH of the silicic acid samples was adjusted to 2.0 with 5 M phosphoric acid. The samples were stirred for 2 h at 4 ºC and heated at 80 ºC for 5 min. After centrifugation (1,000 x g for 20 min), the pH of the supernatants was adjusted to 6.0 with 5 M NaOH, and bacteriocin activity was determined in all samples collected during adsorption and desorption procedures [10, 11]. After that, collected samples were prepared for cation-exchange chromatography (CEC) for further purification.

2.6 Cation-exchange chromatography (CEC)

The bacteriocin preparation (15 ml) was filter-sterilized (0.22 μm pore size). It was then applied to a Whatman carboxymethyl cellulose column CM-52 previously equilibrated with sodium phosphate buffer (50 mM, pH 6.6). The column (30 cm x 2.5 cm) was washed with the same buffer, followed by a 500 ml linear NaCl gradient (0-1 M) in sodium phosphate buffer. Fractions of 5 ml were collected at a flow rate of 1.5 ml min-1, and monitored for absorption at 280 nm. For inhibitory activity against the indicator isolate, *V. parahaemolyticus*, the agar well diffusion method was used. Fractions showing inhibitory activity were pooled, dialyzed against distilled water and freeze dried [10, 11].

2.7 Detection of molecular weight

The molecular weight of bacteriocin was detected by gel filtration method on sephadex G-150 (0.75×100 cm) (Pharmacia Fine Chemical, Sweden) using lysozyme, trypsin inhibitor and α-amylase (Sigma, USA) as reference proteins [12].

2.8 Effect of enzymes

Sensitivity of bacteriocin to different enzymes was determined using purified bacteriocin treated with the following enzymes: pepsin, proteinase K, lipase, α-amylase and DNase (Sigma, USA) before boiling for 2 min. to inactivate the enzymes. After performing each treatment, bacteriocin was tested for antibacterial activity against *V. parahaemolyticus* [13].

2.9 Effect of crude and purified bacteriocin on *V. parahaemolyticus*

Agar well diffusion method was used to detect antibacterial activity of the crude and purified bacteriocin produced by *L. buchneri* against *V. parahaemolyticus* isolates at the concentrations of 1,000 and 500 μg/ml according to Batdorj *et al.* [14].

2.10 Statistical analysis

The intergroup variation was assessed by one way analysis of variance (ANOVA) at 1% level using sigma state statistical software.

3. Results and Discussion

Out of 11 local yoghurt samples, only 3 isolates of *L. buchneri* were found and the isolate No. 3 was the most producer of bacteriocin. Bacteriocin was adsorbed 100% onto silicic acid at pH 6.0-7.0 (Figure 1). Below or above these pH values, adsorption was decreased, ranging between 35 and 90%. Therefore, pH 6.0 was used for the purification procedure. Sixty-five percent of bacteriocin bound silicic acid was desorbed at pH 2.0 with a combination of heat (80 C for 5 min) and NaCl (100 mM). After the silicic acid step, the specific activity increased to 105.92 (Au/mg) and purity of the bacteriocin increased 3.25 fold (Table 1). Yildirim [15] reported that 85% of buchnericin LB bound silicic acid was desorbed at pH 2.0. Janes *et al.* [16] reported that nisin, tetragenocin A and enterocin CS1 showed 90-94% desorption from silicic acid, whereas pediocin RS2 showed only 50% desorption from silicic acid. After adsorption to cation exchange chromatography (CEC), the bound bacteriocin was desorbed from the column by a linear NaCl gradient, yielding a single peak of inhibitory activity.

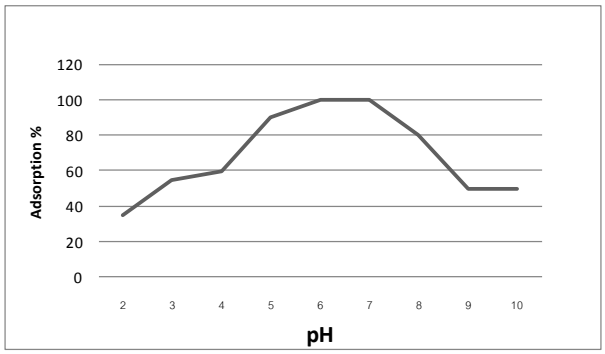

Figure 1. Effect of pH on adsorption of *Lactobacillus buchneri* bacteriocin to silicic acid

The molecular weight was determined to be about 3.4 kDa. The molecular size of buchnericin LB produced by Yildirim and Yildirim [7] was about 3.5-4.5 kDa according to SDS-PAGE. The binding of the bacteriocin to silicic acid and CEC is dependent on the pH. This indicated that *L. buchneri* bacteriocin is a cationic molecule like other lactic acid bacteria bacteriocins [10, 11, 17]. The purification procedure including silicic acid adsorption/desorption and CEC resulted in a 11.11 fold increase in the final specific activity 1176.47 Au/mg of pure bacteriocin compared to the culture supernatant which was 32.64 Au/mg Table 1.

The bacteriocin lost its activity completely after treatment with proteinase K and pepsin; however, it was resistant to non-proteolytic enzymes such as lipase, α- amylase and DNase Table 2. These results indicated that the inhibitory agent is a protein since protease sensitivity is a key criterion for the characterization of a bacteriocin. After treatment with lipase and α- amylase, bacteriocin did not lose its inhibitory activity. These results showed that lipid and carbohydrate moieties were not responsible for its antimicrobial activity.

The results also indicated that bacterioin of both concentrations (500 and 1,000 μg/ml) possesses significant antimicrobial activity against *V. parahaemolyticus* in contrast with control ($P<0.01$) with inhibition zone of (17.44±1.95, 21.98±0.63 mm, prospectively) and the antimicrobial activity of crude and purified bacteriocin at the concentration of 1000 μg/ml was higher than that of the other concentration (500 μg/ml). The antimicrobial activity of purified bacteriocin was significantly higher than that of crude bacteriocin ($P<0.01$) as shown in Table 3. (24.35±1.66, 33.45±2.08)

Table 1. Purification steps of *Lactobacillus buchneri* bacteriocin from isolate No. 3

Purification stage	Volume (ml)	Total activity (Au/ml)[a]	Protein con.(mg/ml)[b]	Specific activity(Au/mg)[c]	Purification fold[d]	Recovery (%)[e]
Culture supernatant	100	7,350	225.2	32.64	1	100
Silicic acid	25	2,680	28.7	105.22	3.25	41.36
CEC	5	800	0.64	1,176.47	11.11	26.32

Note: a, b, c, d and e: These values calculated according to the international methods of protein activity measurement

Table 2. Effect of enzymes on *Lactobacillus buchneri* bacteriocin activity

Enzymes	Activity (%)
Proteinase K	0
α-amylase	100
DNase	100
Pepsin	0
Lipase	100

Table 3. Antibacterial activity of *Lactobacillus buchneri* bacteriocin against *Vibrio parahaemolyticus*

Treatment	Concentration (µg/ml)	Inhibition zone (mm) Mean ±SD
Crude buchnericin LB	500	17.44±1.95[a]
	1,000	21.98±0.63[a]
Purified buchnericin LB	500	24.35±1.66[a]
	1,000	33.45±2.08[a]
Control D.W	0	0±0

Note: [a]Significant differences according to control ($P<0.01$)

4. Conclusions

Bacteriocin was adsorbed 100% onto silicic acid at pH 6.0-7.0. Below or above these pH values, adsorption was decreased, ranging between 35 and 90%. Therefore, pH 6.0 was used for the purification procedure. The molecular weight was determined to be about 3.4 kDa. The bacteriocin lost its activity completely after treatment with proteolytic enzymes and it was resistant to non-proteolytic enzymes. These results indicated that the bacteriocin is a protein. The antimicrobial activity of purified bacteriocin was significantly higher than that of crude bacteriocin.

References

[1] Klaenhammer, T.R., **1993**. Genetics of bactericins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, 12, 39-86.

[2] Nettles, C.G. and Barefoot, S.F., **1993.** Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. *Journal of Food Protection*, 56(4), 338-356

[3] Kleinschmit, D.H., Schmidt, R.J. and Kung, L.**, 2005**. The effects of various antifungal additives on the fermentation and aerobic stability of corn silage. *Journal of Dairy Science*, 88, 2130-2139.

[4] Kung, L. and Ranjit, N.K., **2001**. The effect of *Lactobacillus buchneri* and other additives on the fermentation and aerobic stability of barley silage. *Journal of Dairy Science*, 84, 1149-1155.

[5] Taylor, C.C., Ranjit, N.J., Mills, J.A., Neylon, J.M. and Kung, L., **2002**. The effect of treating whole-plant barley with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for dairy cows. *Journal of Dairy Science*, 85, 1793-1800.

[6] Sumner, S.S., Speckhard, M.W., Somers, E.B. and Taylor S.L., **1985**. Isolation of histamine-producing Lactobacillus buchneri from Swiss cheese implicated in a food poisoning outbreak. *Applied and Environmental Microbiology*, 50, 1094-1096.

[7] Yildirim, Z. and Yildirim, M., **2001**. Characterization of buchnericin LB produced by *Lactobacillus buchneri* LB. *Turkish Journal of Biology*, 25, 73-82.

[8] Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T., **1994**. Bergey's Manual of Determinative Bacteriology. 9th ed. Baltimore, Maryland. USA: Williams and Wilkins Company. http://dx.doi.org/10.1111/j.1365-2672.2006.02966.x

[9] Ogunbanwo, S.T., Sanni, A.I. and Onilude, A.A., **2003**. Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. *African Journal of Biotechnology*, 2(8), 219-227.

[10] Yildirim, Z., Winters, D.K. and Johnson, M.G., **1998**. Gene locus and mode of action of lactococcin R produced *by Lactococcus lactic* sμgsp. cremoris R (a radish isolate). *Annual IFT Meeting*, Atlanta, Georgia.

[11] Yildirim, Z., Winters, D.K. and Johnson, M.G., **1999**. Purification, amino acid and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. *Journal of Applied Microbiology*, 86, 45-54.

[12] Yeasmin, T., Tang, Md. A.K., Razzaque, A. and Absar, N., **2001**. Purification and characterization of three galactose specific lectins from Mulberry seeds (*Morus* sp.). *European Journal of Biochemistry*, 268, 6005-6010.

[13] Mahdi, L.H. and Shafiq, SH.A., **2014**. Effects of crude and purified bacteriocin of *Pediococcus pentosaceus* on the growth and zearalenone production by *Fusarium graminearum*. *International Journal of Current Engineering and Technology*, 4(1), 1-5.

[14] Batdorj, B., Dalgalarrondo, B.M., Choieset, Y., Pedroche, J., Metro, F. and Prevost, H., **2006**. Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. *Journal of Applied Microbiology*, 101, 837-848.

[15] Yildirim, M., **2001**. Purification of buchnericin LB produced by *Lactobacillus buchneri* LB. *Turkish Journal of Biology*, 25, 59-65.

[16] Janes, M.E., Nannapaneni, R. and Johnson, M.G., **1997**. Rice hull ash an adsorbent and delivery agent for. Bacteriocins. *AFT Annual Meeting*, Orlando, Florida June 14-18, Paper 14D-11, p.31.

[17] Bhunia, A., Johnson, M.C., Ray, B. and Kalchanand, N., **1991**. Mode of action pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. *Journal of Applied Bacteriology*, 70, 25-33.