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Abstract: Buchnericin LB produced by *Lactobacillus buchneri* LB was purified to homogeneity by a rapid and simple three-step purification procedure including freeze drying, silicic acid adsorption/desorption and cation-exchange chromatography. After the silicic acid and cation-exchange chromatography steps, the activity of buchnericin LB was recovered by 85 and 25%, and its purity increased about 111 and 2,500 fold, respectively. It was determined that the adsorption of buchnericin LB to silicic acid and cation-exchange chromatography was dependent on the pH of the suspending environment. The molecular weight of buchnericin LB was determined to be about 4.0 kDa by tricine SDS-PAGE.

Key Words: Bacteriocin, buchnericin LB, *Lactobacillus bunchner*i LB, purification, silicic acid, cation-exchange chromatography.

Introduction

Lactic acid bacteria are comprised of at least ten genera according to recent taxonomic revisions: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus* and *Vagococcus* (1). They are widely used as starter cultures in a variety of food fermentations. It is well known that many lactic acid bacteria show antagonistic activities against other bacteria, including food spoilage organisms and food-borne pathogens. There are several different mechanisms responsible for this inhibition. In most cases, the inhibition is caused by the formation of organic acids and hydrogen peroxide and/or bacteriocin production. Bacteriocins are antimicrobial proteinaceous compounds produced by bacteria. Their antimicrobial activity is directed towards sensitive Gram-positive bacteria, some of which are associated with food spoilage and food-borne pathogens (2, 3).

The purification methods are based on observations that the bacteriocin molecules (i) are excreted by the producer cells; (ii) are cationic; (iii) are hydrophobic; (iv) adsorb to the cell surface of the producer cells; and (v) adsorb in a pH-dependent manner, with high adsorption occuring at about pH 6.0 and low adsorption at about pH 2.0 (4). Most approaches to purification are initiated with a method to concentrate bacteriocins from culture supernatants (i.e., reduce working volume). The methods that have proven to simultaneously conctrate

bacteriocins include vacuum concentration, precipitation by salt fractionation, acid preciptation, organic solvent precipitation, freeze drying and ultrafiltration (5, 6, 7). Yang et al. (8) have reported a method for the purification of pediocin involving the adsorption of the bacteriocin onto producer cells at pH 5.5 followed by extraction at pH 2.0. However, the yield of bacteriocin by this procedure is very low (10%). Coventry et al. (9) partially purified nisin, pediocin, brevicin and piscicolin by adsorption onto Micro-Cel (a food grade diatomite calcium silicate anticaking agent) and subsequently desorption. The authors were only able to desorb 75% of bacteriocins with 1% sodium dodecyl sulfate (SDS). This level was very low without using SDS. Janes et al. (10) have reported a purification method involving bacteriocin adsorption/desorption to rice hull ash and silicic acid using pH reduction.

Although the procedures mentioned above play an important role in bacteriocin purification, they typically do not provide for a high degree of resolution from the abundance of contaminating proteins contributed by growth media and/or cellular metabolism. Therefore, several methods of chromatography, including size exclusion (gel filtration), cation exchange, and hydrophobic interaction, have been used to achieve purification of bacteriocins.

Yıldırım (11, 12) has reported a bacteriocin, called buchnericin LB, from *Lactobacillus buchneri* LB. Buchnericin LB inhibits the growth of some selected species of the genera *Listeria, Bacillus, Micrococcus, Enterococcus, Streptococcus, Lactobacillus, Leuconostoc* and *Pediococcus*. It is sensitive to proteolytic enzymes, whereas it is resistant to non-proteolytic enzymes, low and high pH (2.0-9.0), heat (90-121°C for 15 min) or organic solvents.

To investigate the biochemical structure and the potential application of bacteriocins in the most effective way, it is very important to purify the bacteriocins by a simple method. The objectives of this study were to purify buchnericin LB by using silicic acid adsorption/desorption and cation exchange chromatography and to determine its purity.

Materials and Methods

Bacterial culture and preparation of buchnericin LB

To prepare buchnericin LB, *Lactobacillus buchneri* LB was cultured in de Mann Rogosa Sharpe (MRS) broth at 30°C for 18 h, and then cell-free culture supernatant was collected by centrifugation at 1,000 x g for 20 min. After that, it was filter-sterilized (0.45 µm pore size) and freeze dried. *Lb. buchneri* LB was kept in MRS with 20% glycerol at -70°C.

Adsorption and desorption of buchnericin LB onto/from silicic acid

For adsorption of buchnericin LB onto silicic acid (100 mesh), freeze-dried supernatants reconstituted with 150 ml of distilled water were fractionated into 10 ml. Buchnericin LB 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%)

was added into each sample, they were stirred overnight at 4 ºC and then centrifuged at 1,000 x g for 20 min. Buchnericin LB 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 buchnericin LB, the pHs of the silicic acid samples were adjusted to 2.0 with 5 M phosphoric acid. The samples were stirred for 2 h at 4ºC and heated at 80ºC for 5 min. After centrifugation (1,000 x g for 20 min), the pHs of the supernatants were adjusted to 6.0 with 5 M NaOH, and bacteriocin activity was determined in all samples collected during adsorption and desorption procedures (13, 14). After that, collected samples were prepared for cation exchange chromatography for further purification.

Cation-exchange chromatography (CEC)

After buchnericin LB preparation (15 ml) was filter-sterilized (0.22 µm pore size), it was 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 strain, *Lactobacillus plantarum* NCDO 955, the agar spot test was used. Fractions showing inhibitory activity were pooled, dialyzed against distilled water and freeze dried (13, 14).

Tricine SDS-PAGE

Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 16.5 %) was used to determine the purity of buchnericin LB (15, 16). The molecular weight standard was obtained from Bio-Rad.

Estimation of protein concent of the bacteriocin-containing samples was determined by the Lowry method (17) with BSA (brovine serum albumin) (Sigma) as standard.

Results and Discussion

Buchnericin LB was adsorbed 100% onto silicic acid at pHs 5.0-7.0 (Fig. 1). Below or above these pH values, adsorption was decreased, ranging between 70 and 90%. Therefore, pH 6.0 was used for the purification procedure. Eighty-five percent of buchnericin LB bound silicic acid was desorbed at pH 2.0 with a combination of heat (80ºC for 5 min) and NaCl (100 mM). After the silicic acid step, the specific activity and purity of buchnericin LB increased 111 fold (Table 1). Janes et al. (10) 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 buchnericin LB was desorbed from the column by a linear NaCl gradient, yielding a single peak of inhibitory activity.

The peak of buchnericin LB was accompanied by an absorption peak at 280 nm (Fig. 2). Fractions showing inhibitory activity against *Lb. plantarum* were analyzed further by tricine SDS-PAGE, and the purified buchnericin LB showed a single band of protein, which was indicative of high purity (Fig. 3A). Overlaying the stained-destained gel with *Lb. plantarum* showed a clear zone of inhibition (Fig. 3B). The molecular weight was determined to be about 4.0 kDa.

The binding of buchnericin LB to silicic acid and CEC is dependent on pH. This indicates that buchnericin LB is a cationic molecule like other lactic acid bacteria bacteriocins (13, 14, 18). The purification procedure including silicic acid adsorption/desorption and CEC resulted in a 2,500-fold increase in the final specific activity of pure buchnericin LB compared to the crude culture

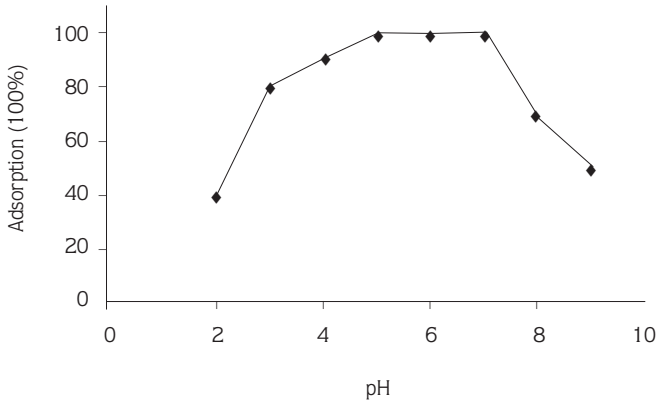

Figure 1. Effect of pH on adsorption of buchnericin LB to silicic acid.

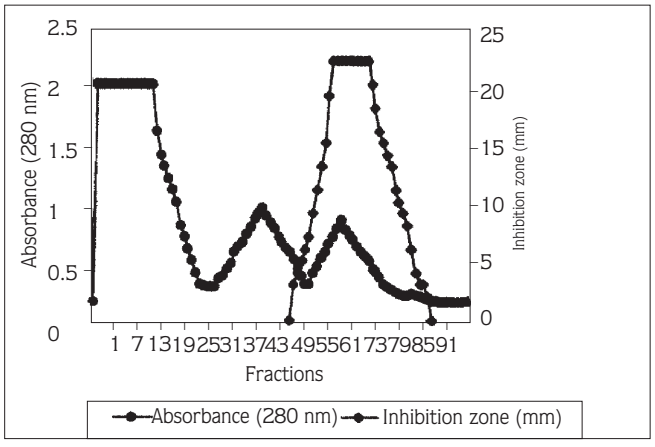

Figure 2. Elution profile of buchnericin LB at 0-1 M NaCl by cation exchange chromatography. The sample (15 ml) applied to the column was desorbed from silicic acid. The starting amount of culture was 1.5 L.

supernatant (Table 1). In addition, the final recovery was 25%. Plantaricin A from *Lb. plantarum* has been purified-phase chromatography (19). The purification resulted in a 1,330-

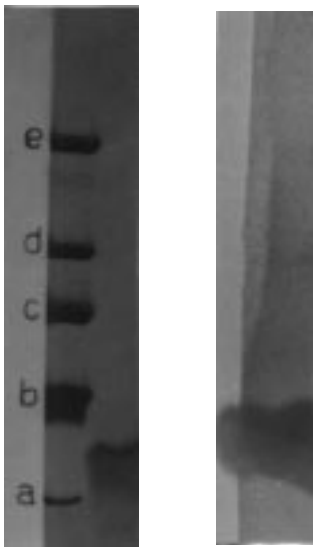

Figure 3. A: gel of purified buchnericin LB obtained by tricine SDS-PAGE electrophoresis. Lane 1, low molecular weight standard (Bio-Rad): a, 1,423; b, 3,496; c, 6,512; d, 14,437; e, 16,950; f, 26,625 Da; lane 2, purficied buchnericin LB. B: gel overlaid with a lawn of *L. plantarum* cells to determine the molecular weight of buchnericin LB.

Table 1. Purification scheme of buchnericin LB.

Volume Sample	Total (ml)	Total Protein (mg)	Specific Activity (AU)[5]	Total Activity (AU/mg)[6]	Recovered (%)	Fold of Purification
CFS[1]	1,500	16,890	4,800,000	284	100	1
FD[2]	150	16,850	4,752,000	282	99	0.99
SC[3]	15	130	4,080,000	31,385	85	111
CEC[4]	1.5	1.7	1,200,000	705,882	25	2,486

[1]Cell free supernatant;
[2]Freeze dried;
[3]Silicic acid;
[4]Cation exchange chromatography
[5]Arbitrary unit (AU/ml) is the reciprocal of the highest dilution in a 10 µl of which gave a clear zone of inhibition on alown of sensitive cells, so
AU= Diameter of inhibition zone x volume of supernatant (ml)/10 µl
[6]Specific activity=Total activity/Total protein x 100.

fold increase in specific activity and a 5% recovery of bacteriocin. Bavaricin MN from *Lb. sake* was purified by ammonium sulphate precipitation, anion exchange and cation exchange chromatography with a 135-fold increase in purity and a 10.7% recovery (20). These results indicate that buchnericin LB was purified by a rapid and simple purification method with high purity and yield.

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