Novel Method To Extract Large Amounts of Bacteriocins from Lactic Acid Bacteria†

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Antimicrobial peptides, bacteriocins, produced by lactic acid bacteria were adsorbed on the cells of producing strains and other gram-positive bacteria. pH was a crucial factor in determining the degree of adsorption of these peptides onto cell surfaces. In general, between 93 and 100% of the bacteriocin molecules were adsorbed at pHs near 6.0, and the lowest (<5%) adsorption took place at pH 1.5 to 2.0. On the basis of this property, a novel isolation method was developed for bacteriocins from four genera of lactic acid bacteria. By using this method we made preparations of pediocin AcH, nisin, sakacin A, and leuconocin Lcm1 that were potent and concentrated. This method produced a higher yield than isolation procedures, which rely on precipitation of the bacteriocins from the cell-free culture liquor. It is simple and can be used to produce large quantities of bacteriocins from lactic acid bacteria to be used as food biopreservatives.

In the search for a food biopreservative, investigations on certain antibacterial proteins (bacteriocins) from lactic acid bacteria have been very popular (11, 18, 25). To study their chemical and antibacterial properties and to determine their effectiveness in food systems, it is necessary to obtain relatively large quantities of these peptides in a pure and concentrated form. Currently, most methods rely on ammonium sulfate precipitation of the bacteriocins from cell-free culture liquor. This method has been used to obtain bacteriocins from Pediococcus spp. (4–6, 13), Lactobacillus spp. (17, 21, 23), Lactococcus spp. (12, 15, 16, 24), and Leuconostoc spp. (10, 14). However, there is general agreement that it does not yield a good product because many other proteins from the medium can also be precipitated and the yield is not very high (5, 9, 12). For further purification of precipitated bacteriocins, especially in the determination of the amino acid composition and sequence, these researchers have used various column chromatography techniques.

Commercial niacin preparations are available in highly purified food-grade form. However, the methods used commercially are not known. Mattick and Hirsch (20) used a combination of acid treatment of the culture followed by removal of the cells and then solvent extraction and precipitation to obtain nisin with high potency. Later, other workers used several different methods based on propanol-NaCl or butanol-acetic acid extraction from culture supernatant (2, 9) and breaking of cells and extraction with acid (1, 31). Although the nisin preparations had high potency, the methods were laborious and total yields were low.

Bhunia et al. (7), while studying the mode of bactericidal action of pediocin AcH from Pediococcus acidilactici H on sensitive cells of Lactobacillus plantarum NCDO 955, observed that adsorption of pediocin AcH to the cells is pH dependent. Maximum adsorption occurred at pH 6.0 to 6.5, but when the pH of the cell suspension was reduced to 2.0 or below, the pediocin AcH was not adsorbed. It is known that, in general, cells of a bacteriocin-producing bacterial strain adsorb the bacteriocin molecules that they produce (7, 13, 18, 25, 30). We hypothesized that if, after fermentation, the culture broth of a producer strain was adjusted to the pH for maximum adsorption of the bacteriocin onto the cell surfaces, the cells with adsorbed bacteriocin molecules could easily be removed from the culture liquor by centrifugation. The peptide could then be selectively released from the cells at pH 1.5 to 2.0, and the preparation could provide large quantities of purified bacteriocin. This hypothesis was tested with bacteriocin-producing strains from four genera of lactic acid bacteria and the results are presented here.

MATERIALS AND METHODS

Bacterial cultures and media. The bacterial strains used in this study are described in Table 1. Pediococcus acidilactici LB 42-923 (27), used to produce pediocin AcH, was grown at 37°C for 18 h in TGE broth (8). Lactococcus lactis subsp. lactis ATCC 11454, used to produce nisin, was grown at 30°C for 24 h in TGE buffered broth, which consisted of TGE broth plus 0.5% sodium citrate, 0.1% sodium acetate, and 0.05% dipotassium phosphate (pH 6.5). Leuconostoc carnosum LM1, used to produce leuconocin Lcm1, and Lactobacillus sake LB 706, used to produce sakacin A (29), were grown at 30°C for 24 h in MRS broth (Difco). All indicator bacteria were grown in TGE broth and transferred daily.

Antimicrobial activity assay of bacteriocin. Each bacteriocin preparation was serially diluted (1:10, 1:20, 1:30, etc.), and 5 μl from each dilution was spotted onto a lawn of appropriate indicator bacteria. Activity units (AU) per milliliter were calculated as previously reported (7, 8). The indicator bacteria were Lactobacillus plantarum NCDO 955 for pediocin AcH and nisin, Leuconostoc mesenteroides Ly for sakacin A, and Enterococcus faecalis MB1 for leuconocin Lcm1.

Preparation of cells for bacteriocin adsorption. A desired bacterial strain (Fig. 1) was grown overnight in the appropriate medium, and the cells were collected by centrifugation, washed in 1/5 volume of sterile 5 mM sodium phosphate (pH 6), resuspended in 1/5 vol of sterile 1.0 M NaCl solution (pH 2.0, adjusted with 5% phosphoric acid), and mixed at 4°C for 1 h. The cells were harvested by centrifugation,
washed once with sterile deionized water (dH₂O), and resuspended in sterile dH₂O to 20 times the original culture volume.

**Influence of pH on bacteriocin adsorption by producing cells and indicator cells.** A cell suspension of a desired bacterial strain (0.1 ml, about 10⁷ cells) and a bacteriocin preparation (0.1 ml, about 10⁶ AU) were added to 1.8 ml of 5 mM sodium phosphate adjusted to a pH range from 1.5 to 10.0 (with phosphoric acid and NaOH). This mixture was incubated at 4°C for 30 min and centrifuged at 17,000 × g for 5 min, and the AU of the bacteriocin in the supernatant was assayed (7, 8). Control I consisted of 0.1 ml of dH₂O instead of bacteriocin, and control II had 0.1 ml of dH₂O instead of cell suspension. The percentage of bacteriocin adsorbed was calculated as follows: percentage of bacteriocin adsorbed = 100 × [1 - (AU/ml in cell-free supernatant - AU/ml in control I)]/AU/ml in control II).

**Extraction of adsorbed bacteriocin from producer cells.** For extraction of pediocin ACH, nisin, leuconocin Lcm1, and sakacin A, each producer strain was grown to early stationary phase (about 5 × 10⁸ cells per ml) in 1 liter of the appropriate medium. The culture broths were adjusted to pH 6.5 for pediocin ACH, nisin, and sakacin A and pH 5.5 for leuconocin Lcm1. Each culture broth was heated to 70°C for 25 min to kill the cells (heating can be done before pH adjustment also), and the cells were harvested by centrifugation at 15,000 × g for 15 min. Supernatant activity was assayed to calculate the amount of bacteriocin not adsorbed onto the cells. After the cells had been washed with 5 mM sodium phosphate (pH 6.5), they were resuspended in 50 ml of 100 mM NaCl at pH 2.0 (adjusted with 5% phosphoric acid) for pediocin ACH, leuconocin Lcm1, and sakacin A and pH 2.5 for nisin and mixed with a magnetic stirrer for 1 h at 4°C. Cell suspensions were then centrifuged at 29,000 × g for 20 min, and the cells were resuspended in 5 mM sodium phosphate (pH 6.5) and assayed to calculate the amount of bacteriocin lost with the cells. The supernatants were dialyzed in 1,000-molecular-weight-cutoff dialysis bags against dH₂O at 4°C for 24 h and then freeze-dried. The total dry matter, protein concentration (19), and bacteriocin activity of each product were determined. This process was performed twice for each bacteriocin.

**SDS-PAGE and identification of the activity band.** Freeze-dried extraction preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Methods previously reported (4, 5) were used with a Tris-tricine system (28). This method used a 10-cm layer of 16.5% acrylamide resolving gel and a 2-cm layer of 10% acrylamide spacer gel. The electrophoresis was run at 15°C and 20 mA for the first 2 h and then at 30 mA for another 12 h. Sample preparation, application of samples, and gel division after electrophoresis have been described before (4). One-half of each gel was stained with Coomassie brilliant blue, and the other half was used for identification of the bacteriocin band by using growth inhibition of *E. faecalis* MB1 (4). For growth inhibition, the gel half was washed in deionized water for 2 h at 25°C, placed on a TGE agar prepared and overlaid with soft agar seeded with *E. faecalis* MB1. The plate was incubated at 30°C for 24 h, examined for the location of the zone of growth inhibition, and photographed. The stained gel half was destained and photographed.

**RESULTS**

**Sensitivity of bacterial strains to bacteriocins.** The four bacteriocin-producing strains and the three indicator strains were tested for sensitivity to four bacteriocins (Table 1). Their sensitivities varied greatly: *Lactococcus lactis* subsp. *lactis* ATCC 11454 was not sensitive to any bacteriocin, whereas *E. faecalis* MB1 was sensitive to all four. These data allowed us to select a specific strain for measuring the AU of each bacteriocin preparation and for measuring, in separate experiments, the adsorption of each bacteriocin to a sensitive strain and the producer strain (Fig. 1).

**Effect of pH on adsorption of bacteriocin onto producer and indicator cells.** The influence of pH on the adsorption of pediocin ACH, nisin, sakacin A, and leuconocin Lcm1 onto their respective producer and indicator strains is presented in Fig. 1. Adsorption of all four bacteriocins onto cells was strongly influenced by the pH of the suspending environment. Pediocin ACH was adsorbed 100% at pH 6.0 to 6.5, while at pH below 1.5 it was not adsorbed to either *P. acidilactici* LB 42-923 or *Lactobacillus plantarum* NCDO 955. Maximum adsorption of nisin to both producer and indicator bacteria occurred at pH 6.5, and complete loss of adsorption was found at pH 3.0 and below. Maximum sakacin A adsorption occurred at pH 5.5 and above on both the producer and indicator strains. At pH 2.0 no sakacin A was adsorbed to the producer cells but about 75% remained adsorbed onto *E. faecalis* MB1. Leuconocin Lcm1 had a sharp adsorption curve on producer cells, with a maximum

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**TABLE 1. Bacterial strains, bacteriocin sensitivity, and growth conditions**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacteriocin produced</th>
<th>Bacteriocin sensitivity</th>
<th>Growth medium</th>
<th>Growth conditions</th>
<th>Source (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acidilactici</em> LB 42-923</td>
<td>Pediocin ACH</td>
<td>Nisin</td>
<td>TGE</td>
<td>37°C, 20 h</td>
<td>Our laboratory (26)</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> ATCC 11454</td>
<td>Nisin</td>
<td>None*</td>
<td>TGE</td>
<td>30°C, 24 h</td>
<td>ATCC*</td>
</tr>
<tr>
<td><em>L. sake</em> LB 706</td>
<td>Sakacin A</td>
<td>Nisin</td>
<td>MRS</td>
<td>25°C, 24 h</td>
<td>F. K. Lucke (28)</td>
</tr>
<tr>
<td><em>L. casei</em> NCDO 300</td>
<td>Leuconocin Lcm1</td>
<td>Pediocin ACH, nisin</td>
<td>TGE</td>
<td>30°C, 18 h</td>
<td>Our laboratory</td>
</tr>
<tr>
<td><em>L. mesenteroides</em> NCDO 685</td>
<td>None</td>
<td>Pediocin ACH, nisin, sakacin A</td>
<td>TGE</td>
<td>30°C, 18 h</td>
<td>Laboratory (7)</td>
</tr>
<tr>
<td><em>E. faecalis</em> MB1</td>
<td>None</td>
<td>Pediocin ACH, nisin, sakacin A, leuconocin Lcm1</td>
<td>TGE</td>
<td>30°C, 18 h</td>
<td>Laboratory*</td>
</tr>
</tbody>
</table>

* Not sensitive to any of the bacteriocins tested.
* ATCC, American Type Culture Collection, Rockville, Md.
* Isolated from processed meat products in our laboratory.
* Nonbacteriocin producer strains were used as indicators.
* Originally from the Microbiology Department, University of Wyoming.
Extraction and purification of bacteriocin. On the basis of the influence of pH on the adsorption and release of each bacteriocin, an extraction method for bacteriocin was developed. The recovery and loss of each bacteriocin at different stages of purification and in the final dried product are presented in Table 2. The loss of bacteriocin in the supernatant following harvesting of the cells ranged from 0 to 7.7%, and loss with the cells after extraction ranged from 0.6 to 2.3%. Recovery in dried preparations ranged from 44.3% for sakacin A to 106.7% for pediocin AcH. The potency (AU per gram) of bacteriocins ranged from $1.8 \times 10^4$ to $5.0 \times 10^2$ on a dry-matter basis and from $1.1 \times 10^3$ to $4.9 \times 10^4$ on a protein content basis (19). Although the loss of sakacin A in supernatant and cells was very low, the total yield in the dried preparation was only 44%. It was not clear why there was such a discrepancy. The yields of dry matter and protein for both Lactobacillus sake Lb 706 and Leuconostoc carnosum Lm1 were higher than for P. acidilactici LB 42-923 and Lactococcus lactis subsp lactis ATCC 11454.

SDS-PAGE analysis of the preparations. A Coomassie blue-stained SDS-PAGE gel revealed a single sharp band from both the pediocin AcH and nisin preparations, especially when they were tested within 2 weeks of preparation (Fig. 2). After storage for 2 weeks or more, pediocin AcH and nisin preparations produced more than one band. However, the sakacin A and leuconocin Lcm1 preparations produced several bands, some of which were diffused. A comparison of the bands in the stained gel with the gel half showing zones of inhibition indicated that the lowest band for each preparation contained the active bacteriocin (Fig. 2 and 3). Nisin, sakacin A, and leuconocin Lcm1 bands were very close to 2.5 kDa, while pediocin AcH was just above the 2.5-kDa standards. Also, leuconocin Lcm1 gave two activity bands, one just below the other, and the pediocin AcH preparation showed a streak of slight activity above the main zone (Fig. 3).

DISCUSSION

Adsorption of bacteriocin molecules by the Pediococcus (7, 13), Lactobacillus (18), Lactococcus (16), and Leuconostoc (10, 14) producer strains, as well as by other sensitive and resistant gram-positive bacteria, has been reported. Also, the pH of optimum adsorption for nisin (16) and pediocin AcH (7) by gram-positive bacteria has been reported. Bhunia et al. (7) recently reported the conditions for adsorption of pediocin AcH onto sensitive Lactobacillus plantarum NCDO 955. This report was the basis for the present study on the influence of pHs between 1 and 10 on the level of adsorption of four bacteriocins from four genera of lactic acid bacteria onto the respective producer strains and lactic acid bacterium strains sensitive to a particular bacteriocin. For this purpose Lactobacillus plantarum NCDO 955 was used for pediocin AcH and nisin, Leuconostoc mesenteroides Ly was used for sakacin A, and E. faecalis MB1 was used for leuconocin Lcm1 (Table 1; Fig. 1).

Methods for bacteriocin isolation that involved removal of cells from culture broth, following adjusting the pH to 6.0 (13), and/or precipitation of cell-free supernatant with (NH₄)₂SO₄ will recover only a portion of the total bacteriocin present in the culture broth, because the portion adsorbed on the cells is lost. Since adsorption of bacteriocins is not affected by heating the cells, we used heat-killed cells to avoid any loss of activity by proteolytic enzymes of the cells (7, 8). Although the maximum and minimum adsorption with

![Graphs showing adsorption of bacteriocins](http://aem.asm.org/Downloaded from http://aem.asm.org/ on October 13, 2017 by guest)
respect to pH varied slightly among the bacteriocins, a single protocol could usually be used, namely pH 6.0 for high adsorption onto the cells and pH 2.0 for maximum release from the cells. Extraction at alkaline pH was not used because it is known that nisin (16), pediocin AcH (7), and some other bacteriocins of lactic acid bacteria are inactivated at alkaline pHs. Phosphoric acid was used to adjust the mixtures to the desired pH; other acids could also probably be used. We used 100 mM NaCl during extraction to prevent the bacteriocin molecules from clumping and thus being removed with the cells during centrifugation.

The total recoveries of pediocin AcH, nisin, and leuconocin Lcm1 in the dried preparations were relatively high. For pediocin AcH the recovery was over 100%. This may be a result of the dilution method used for the assay of AU. Because only fixed dilutions are used, there may be differences in the end point, which may introduce some error. However, for all three bacteriocins the recovery was over 90%. For sakacin A the recovery was only 44.0%, even though losses in supernatant and cells were very low (Table 2). The reason for this discrepancy is now being investigated. The dried preparations have very high bacteriocin activity based on either dry-matter or protein concentration. Dry-matter and protein concentrations of both sakacin A and leuconocin Lcm1 were relatively high compared with those of pediocin AcH and nisin. Thus, it is possible that *Lactobacillus sake* Lb 706 and *Lactococcus lactis* strain MB1 have some other proteins that were extracted along with the bacteriocins. Some *Lactobacillus* strains have been shown to possess easily extracted surface proteins (3, 26).

The method described here produced dry preparations of bacteriocins that were not only highly potent but, for pediocin AcH and nisin, also pure (Fig. 2). In a separate study the pediocin AcH band from the gel was transferred to a membrane by transblot and the membrane was used for partial sequencing of amino acids (22). The formation of more than one band by stored preparations of pediocin AcH is now being studied. Both sakacin A and leuconocin Lcm1 gave several bands with higher Mr than the bacteriocin. These bands are probably surface or cell wall proteins; this is currently being investigated. In gel overlays there was a single inhibition zone for each bacteriocin, except for leuconocin Lcm1, which gave two zones, suggesting that both monomer and dimer forms of leuconocin Lcm1 were present (Fig. 3).

The actual Mr of the bacteriocins as determined from the amino acid sequences and the Mr of 2,500, as estimated on the basis of their migration in relation to standards in the gel could be quite different. Nisin, with a known Mr of 3,500 (16), forms a band in the same line as the Mr of 2,500. Pediocin AcH, with a known Mr of 4,600 (22), forms a band slightly above 2,500.

### TABLE 2. Recovery of bacteriocins at different stages of extraction and purification

<table>
<thead>
<tr>
<th>Bacteriocin recovered</th>
<th>Pediocin AcH</th>
<th>Nisin</th>
<th>Sakacin A</th>
<th>Leuconocin Lcm1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth (1 liter)*</td>
<td>3.0 x 10⁷ (100%)</td>
<td>3.0 x 10⁷ (100%)</td>
<td>7.0 x 10⁷ (100%)</td>
<td>2.6 x 10⁷ (100%)</td>
</tr>
<tr>
<td>Lost in broth supernatant*</td>
<td>8.0 x 10⁴ (2.7%)</td>
<td>2.0 x 10⁴ (0.7%)</td>
<td>4.0 x 10⁴ (0.6%)</td>
<td>6.0 x 10⁴ (2.3%)</td>
</tr>
<tr>
<td>Lost with cells after extraction*</td>
<td>6.0 x 10⁵ (2.0%)</td>
<td>2.0 x 10⁴ (0.7%)</td>
<td>4.0 x 10⁴ (0.6%)</td>
<td>6.0 x 10⁴ (2.3%)</td>
</tr>
<tr>
<td>In total dried preparation*</td>
<td>3.2 x 10⁵ (106.7%)</td>
<td>2.8 x 10⁴ (93.3%)</td>
<td>3.1 x 10⁵ (44.3%)</td>
<td>2.5 x 10⁵ (96.2%)</td>
</tr>
<tr>
<td>AU/g of dry matter*</td>
<td>5.0 x 10⁸</td>
<td>5.0 x 10⁴</td>
<td>3.1 x 10⁷</td>
<td>1.8 x 10⁷</td>
</tr>
<tr>
<td>AU/g of protein*</td>
<td>4.1 x 10⁴</td>
<td>4.9 x 10⁴</td>
<td>1.5 x 10⁸</td>
<td>1.1 x 10⁸</td>
</tr>
</tbody>
</table>

* Total AU in relation to 1-liter culture broths and supernatants and cells from the broths. Percentages are based on AU in culture broths as 100%.

* AU in freeze-dried products was determined by resuspending the dry material in 50 mM SDS for better dispersion and prevention of clumping of bacteriocin molecules.

* After freeze-drying the values for total dry matter and total protein content, respectively, were as follows: pediocin AcH, 640 and 78 mg; nisin, 560 and 57.5 mg; sakacin A, 956 and 206 mg; leuconocin Lcm1, 1,400 and 230 mg. The results are the average of two extractions.

**FIG. 2.** SDS-PAGE gel half, stained with Coomassie blue. (A) Lanes: 1, Mr standards (a, Mr, 16,900; b, Mr, 14,400; c, Mr, 8,200; d, Mr, 6,200; e, Mr, 2,500); 2, pediocin AcH (old preparation); 3, nisin; 4, sakacin A; 5, leuconocin Lcm1. (B) Lanes: 1, pediocin AcH (fresh preparation); 2, Mr standards. Arrows show respective bacteriocin bands.

**FIG. 3.** SDS-PAGE gel half, showing the zone of growth inhibition of *Escherichia coli* by the bacteriocin bands. Lanes: A, pediocin AcH (old preparation, showing a slight streak of activity above the main zone); B, nisin; C, sakacin A; D, leuconocin Lcm1. These zones of inhibition correspond to the respective bacteriocin bands in Fig. 1A. For further explanation, see the text.
The method described here for the extraction of bacteriocins from lactic acid bacterial strains produces products with high potency and in concentrated form. The total loss is quite low. This method could be an economical procedure to produce large quantities of bacteriocins from lactic acid bacteria for use as food biopreservatives. The extraction procedure described here can also be used for the extraction of proteins from biological systems in which adsorption to and release from specific receptors are pH dependent.

ACKNOWLEDGMENTS

This investigation was partially funded by the National Live Stock and Meat Board, Binational Agricultural Research Development (with Israel), and Wyoming Experimental Station funds.

REFERENCES