Purification and Some Properties of Acidocin 8912, a Novel Bacteriocin Produced by Lactobacillus acidophilus TK8912

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Acidocin 8912, a bacteriocin produced by Lactobacillus acidophilus TK8912, was purified by ammonium sulfate fractionation and successive chromatographies on CM-cellulose, Sephadex G-50, Sephadex G-25, and reversed-phase HPLC on Aquagel RP-300. The purified acidocin 8912 migrated as a single band on SDS-PAGE. The molecular weight was estimated to be 5200 by SDS–PAGE, and 5400 by HPLC gel filtration on TSKgel G3000PWXL. Both the amino acid composition and the N-terminal amino acid sequence analysis indicated that acidocin 8912 was a peptide composed of presumably 50 amino acids containing a Lys residue at the N-terminus. The purified acidocin 8912 showed a bactericidal effect on sensitive cells but not a bacteriolytic effect.

Bacteriocins are proteins with a narrow spectrum of bactericidal activity against species of bacteria closely related to the producer.1) It is known that a number of strains of Lactobacillus acidophilus produce bacteriocins.2,3) Barefoot and Klaenhammer found that a majority (63%) of the L. acidophilus strains they examined produced bacteriocins.4) Nevertheless, little is known about their chemical composition and structure, mode of action, or genetics. Only one bacteriocin from L. acidophilus 11088, lactacin F, has been studied in detail.5,6) and the gene encoding this bacteriocin was recently cloned and sequenced.7) We described in this report the antibacterial activity, the purification, and the structure analysis of a bacteriocin, designated acidocin 8912, which was produced by L. acidophilus TK8912.8,9)

Materials and Methods

Bacterial strains and media. The acidocin 8912 producer Lactobacillus acidophilus TK8912 and the sensitive indicator Lactobacillus casei subsp. rhamnosus ATCC 7469 used in this study were described previously.9) These strains were maintained and grown on MRS medium.9)

Assay for acidocin 8912 activity. Acidocin 8912 activity was measured by the agar-well diffusion method.8) Portions (100 μl) of serial dilutions of samples were added to wells 1 cm in diameter cut into a plate, which was inoculated with indicator cells of L. casei subsp. rhamnosus ATCC 7469, and the plate was incubated. One activity unit (AU/ml) was defined as the reciprocal of the highest dilution giving growth inhibition of the indicator cells.

Measurement of protein. Absorbance at 220 nm was used to monitor the protein peaks on the column chromatographies. The proteins were measured by the method of Lowry et al.10) using bovine serum albumin as the standard protein.

Purification of acidocin 8912. All purification procedures were done at about 4°C unless otherwise stated.

Step 1. Ammonium sulfate fractionation. A 5% transfer from an overnight culture was made into 500 ml of MRS broth. The cells were grown at 30°C to mid-log phase, and the bacteria were removed by centrifugation at 8,000 × g for 30 min. The culture supernatant obtained was heat-treated at 80°C for 20 min. After cooling, ammonium sulfate was added to 30% saturation and the precipitate thus formed was removed, followed by further addition of the salt up to 80% saturation. The precipitate obtained was collected and dissolved with a small amount of distilled water. The solution was dialyzed in no. 3 Spectrapor tubing (Spectrum Medical Industries, Inc.) against distilled water. The dialysate obtained was used as the solution of the crude acidocin 8912.

Step 2. CM-cellulose column chromatography. The solution of the crude acidocin 8912 was put on a CM-cellulose column (2.5 × 30 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.5). After washing the column with 0.1 M sodium borate buffer (pH 9.5) containing 0.1 M KCl, acidocin 8912 was eluted with 0.1 M sodium borate (pH 10.2) containing 0.1 M KCl and 50% acetone. The active fractions were combined and concentrated to half of its original volume by a rotary evaporator at below 37°C. Thus, the solution obtained was dialyzed as before and concentrated.

Step 3. Sephadex G-50 column chromatography. The concentrated solution from step 2 was put on a Sephadex G-50 column (1.5 × 90 cm) equilibrated with 25 mM sodium phosphate buffer, pH 7.5 (buffer A) and eluted with the same buffer. The active fractions were combined and concentrated.

Step 4. Sephadex G-25 column chromatography. The concentrated solution obtained from step 3 was put on a Sephadex G-25 column (1.5 × 90 cm) equilibrated with buffer A and eluted with the same buffer. The active fractions were combined and concentrated.

Step 5. High-performance liquid chromatography. The concentrated solution obtained from step 4 was put on a reversed-phase C-8 column (2.1 × 220 mm, Aquagel RP-300) equilibrated with 0.1% trifluoroacetic acid (TFA). The elution was done at room temperature with a linear gradient from 0 to 70% acetonitrile in 0.1% TFA at a flow rate of 0.2 ml/min.

HPLC gel filtration. Estimation of the molecular weight of the purified acidocin 8912 by HPLC gel filtration was done on a column (7.5 × 300 mm) of TSKgel G3000PWXL with 45% acetonitrile solution containing 0.1% TFA at a flow rate of 0.3 ml/min.

Electrophoresis and assay for acidocin 8912 activity in SDS-polyacrylamide gel. The molecular weight was also estimated using SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli.11) An SDS-polyacrylamide gradient slab gel (4–20%, Bio-Rad) was used. Proteins were stained with a silver stain kit (Wako).

For the activity measurement of acidocin 8912 in an SDS-polyacrylamide gel, the slab gel was washed as described by Bhunia et al.12) Overlay agar was mixed with the indicator cells, and poured onto the gel. After 16 hr of incubation at 37°C, the plate was examined for inhibition zones of the growth of the indicator bacteria.

Amino acid composition and sequence of acidocin 8912. The amino acid

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analysis of the purified acidocin 8912 was done with a JEOL JLC-300 amino acid analyzer. About 500μg of the purified acidocin 8912 was hydrolyzed with 6N HCl at 110°C for 24, 48, and 72 hr in a sealed, evacuated tube. Values for threonine and serine were calculated by extrapolation of data at 24, 48, and 72 hr to zero time; 72 hr values were used for isoleucine and valine. For the analysis of tryptophan, acidocin 8912 was hydrolyzed with 3M p-toluene sulfonic acid containing 0.2% 3-(2-aminoethyylimidole) at 110°C for 24 hr in a sealed, evacuated tube. Cysteine was measured as cysteic acid after performic acid oxidation. Other values were averages of hydrolysates at 24, 48, and 72 hr.

The N-terminal amino acid sequence of acidocin 8912 (about 100μg) was analyzed on a gas phase sequencer (Applied Biosystems model 474A).

**Antibacterial activity.** Cells of a log-phase culture of *L. casei* subsp. *rhamnosus* ATCC 7469 were washed and suspended in 25mM sodium phosphate buffer (pH 7.5) to a final concentration of 10^6 CFU/ml. To this cell suspension, the purified acidocin 8912 was added in amounts giving a final concentration of 0 and 1000 AU/ml. The samples were incubated at 37°C, and then optical density at 600 nm and viable cell counts (CFU/ml) were determined after 0, 5, 15, and 30 min.

**Materials.** CM-cellulose was purchased from Wako Pure Chemical Industries, Ltd., Sephadex G-25 and G-50 were from Pharmacia LKB Biotechnology Inc., Aquapore RP-300 was from Applied Biosystems, and TSKgel G3000PWxl was from Tosoh Co., Ltd. The standard marker proteins for HPLC and SDS-PAGE were from Sigma Chemical Co. All other chemicals were of analytical grade and obtained from commercial sources.

### Results

#### Purification of acidocin 8912

The results of the purification process are summarized in Table I. To inactivate any heat-labile enzyme that might reduce the recovery of acidocin 8912, the culture supernatant was heat-treated, followed by the saturation with ammonium sulfate. The crude concentrate obtained was chromatographed on CM-cellulose. At this stage, the majority of medium contaminants emerged in the run-off fractions and the retained acidocin 8912 in the column was effectively removed from the resin with 50% acetone as the solvent for elution. Active fractions were successively chromatographed on a column of Sephadex G-50, and then on a column of Sephadex G-25. Reversed-phase HPLC, the final step of purification (Fig. 1), gave a single symmetrical peak of the acidocin 8912 activity superimposable on a major protein peak, which was recovered at about 55% acetonitrile. The overall purification procedure resulted in about 2870-fold purification with a yield of 12%, estimated on the basis of the acidocin 8912 activities.

#### Molecular weight

The molecular weight of the purified acidocin 8912 was estimated to be 5400 by HPLC gel filtration (Fig. 2). Furthermore, the zone of growth inhibition of the indicator cells corresponded with the band in the stained gel with a molecular weight of 5200 (Fig. 3). Thus, acidocin 8912 appears to be a monomeric structure.

#### Amino acid composition and N-terminal amino acid sequence

The amino acid composition of acidocin 8912 is given in Table II, assuming the molecular weight of acidocin 8912 to be 5400. No cysteine residues were found after performic acid oxidation of acidocin 8912.

The sequence of 24 consecutive N-terminal amino acid residues of acidocin 8912 was identified as follows; NH2-Lys-Thr-His-Tyr-Pro-Thr-Asn-Ala-X-Lys-Ser-Leu-Arg-Lys-Gly-Phe-X-Glu-Ser-Leu-Arg-X-Thr-Asp, X represents an unidentified residue.

### Table I. Purification of Acidocin 8912 Produced by *L. acidophilus* TK8912

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (AU)</th>
<th>Specific activity (AU/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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</thead>
<tbody>
<tr>
<td>Culture supernatant (500ml)</td>
<td>7,100</td>
<td>57,000</td>
<td>8.03</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate</td>
<td>1,360</td>
<td>38,800</td>
<td>28.53</td>
<td>68.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Cation-exchange chromatography</td>
<td>1.75</td>
<td>14,160</td>
<td>8.09</td>
<td>24.8</td>
<td>1,008</td>
</tr>
<tr>
<td>Gel filtration chromatography (G-50)</td>
<td>1.08</td>
<td>9,780</td>
<td>9.056</td>
<td>17.2</td>
<td>1,128</td>
</tr>
<tr>
<td>Gel filtration chromatography (G-25)</td>
<td>0.58</td>
<td>7,760</td>
<td>13.379</td>
<td>13.6</td>
<td>1,666</td>
</tr>
<tr>
<td>Reversed-phase chromatography</td>
<td>0.31</td>
<td>7,160</td>
<td>23,097</td>
<td>12.6</td>
<td>2,876</td>
</tr>
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</table>

### Fig. 1. Reversed-phase HPLC of Acidocin 8912.

The samples from Sephadex G-25 column (step 4) were put on a 2.1 x 220 mm column of Aquapore RP-300 equilibrated with 0.1% trifluoroacetic acid (TFA). The starting buffer was 0.1% TFA and the limiting buffer was 0.1% TFA, 70% acetonitrile. The flow rate was 0.2 ml/min, and 0.5 ml fractions were collected. After chromatography, acidocin 8912 activities were measured.

### Fig. 2. Estimation of the Molecular Weight of Acidocin 8912 by HPLC Gel Filtration on TSKgel G3000PWxl.

The molecular weight markers used were; (1) cytochrome c, horse heart (12,400), (2) aprotinin, bovine lung (6500), (3) α-melanocyte stimulating hormone (1665), and (4) bradykinin (1060). The closed circle indicates the position of acidocin 8912.
Fig. 3. SDS-Polyacrylamide Gel Electrophoresis (A) and Detection of Acidocin 8912 Activity in an SDS-Polyacrylamide Gel (B).

(A): A total of 2 μl of the purified acidocin 8912 from the reversed-phase HPLC fraction was put on a 4—20% continuous gradient slab gel. After electrophoresis, the gel was silver stained. As molecular weight standards, MW-SDS-17S (Sigma) was used. (B): After electrophoresis, the gel was washed by the method of Bhuinia et al.12 and overlaid with an MRS agar containing L. casei subsp. rhamnosus ATCC 7469 cells to confirm this single band having the inhibition activity.

Table II. Amino Acid Composition of Acidocin 8912

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol (%)</th>
<th>Number of residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td></td>
<td>3.98</td>
</tr>
<tr>
<td>Thr</td>
<td>5.75</td>
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</tr>
<tr>
<td>Ser</td>
<td>10.21</td>
<td>5.11</td>
</tr>
<tr>
<td>Gin</td>
<td>12.45</td>
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<td>Pro</td>
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<tr>
<td>Gly</td>
<td>12.07</td>
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<tr>
<td>Ala</td>
<td>8.39</td>
<td>4.20</td>
</tr>
<tr>
<td>Val</td>
<td>3.99</td>
<td>2.00</td>
</tr>
<tr>
<td>Met</td>
<td>2.07</td>
<td>1.04</td>
</tr>
<tr>
<td>Leu</td>
<td>3.41</td>
<td>1.71</td>
</tr>
<tr>
<td>Tyr</td>
<td>5.48</td>
<td>2.74</td>
</tr>
<tr>
<td>Phe</td>
<td>2.10</td>
<td>1.05</td>
</tr>
<tr>
<td>Lys</td>
<td>2.49</td>
<td>1.25</td>
</tr>
<tr>
<td>His</td>
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</tr>
<tr>
<td>Arg</td>
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</tr>
<tr>
<td>Trp</td>
<td>7.80</td>
<td>3.90</td>
</tr>
</tbody>
</table>

100.00  50

The amino acid composition of the purified acidocin 8912 was calculated assuming the molecular weight to be 5400 estimated by HPLC gel filtration.

Antibacterial action of acidocin 8912

In order to find whether acidocin 8912 had a bactericidal or bacteriolytic mode of action, the effects of the purified acidocin 8912 on viability and lysis of the indicator cells were examined. As shown in Fig. 4, addition of acidocin 8912 resulted in a rapid death of the indicator cells, while the optical density of the cell suspension was unchanged at least after 30 min, suggesting that acidocin 8912 had a bactericidal action without causing concomitant cell lysis.

Discussion

In this study, it was found that acidocin 8912 was a single peptide with a molecular weight of 5400 (Fig. 2) composed of presumably 50 amino acids (Table II). The purified acidocin 8912 showed a bactericidal activity against the sensitive cells (Fig. 4), in accordance with other bacteriocins reported in lactobacillus strains.5,13,14 In contrast, this bacteriocin has a molecular weight distinct from those of other lactobacillus strains as follows: lactacin B, 6,50013; lactocin 27, 12,40013; helveticin, 37,00014; and casein, 40,000.16 Furthermore, the N-terminal peptide sequence of acidocin 8912 does not share any homology to bacteriocins so far isolated from lactic acid bacteria.7,17—19 From these results, acidocin 8912 is different from these bacteriocins.

Cloning of several bacteriocin genes from lactic acid bacteria has been reported.20,21 Recently, four structural genes encoding bacteriocin were sequenced.7,18,22—25 We have previously demonstrated that the genetic determinants of acidocin 8912 production and host immunity in L. acidophillus TK8912 were associated with a plasmid DNA of this strain.26 The partial amino acid sequence of acidocin 8912 should facilitate cloning and DNA sequencing of the gene(s) encoding this bacteriocin.

References

12) A. K. Bhunia, M. C. Johnson, and B. Ray, J. Ind. Microbiol., 2,
Purification of Acidocin 8912 from \textit{L. acidophilus} TK8912


