Isolation and Characterization of a Highly Hydrophobic New Bacteriocin (Gassericin A) from Lactobacillus gasseri LA39

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A new bacteriocin, gassericin A, was purified from the culture fluid of Lactobacillus gasseri LA39 mainly by reverse-phase (RP) chromatography. The purification of gassericin A from a modified MRS broth, in which TWEEN 80 had been replaced by oleic acid, resulted in a 4500-fold increase in specific activity with a 6% recovery. Gassericin A was eluted as a single peak on the chromatogram from RP-HPLC and migrated by SDS–PAGE as a single band with a molecular weight of ca. 3.8 kDa. Gassericin A, a highly hydrophobic bacteriocin, was slightly soluble in water, but its solubility was increased by adding alcohol and acetonitrile. An amino acid analysis revealed that it was composed of 45.7% hydrophobic amino acids in the total residues of 35 amino acids. Gassericin A produced in the MRS broth associated strongly with Tween 80, although several further trials of dissociation were unsuccessful.

Their beneficial role has resulted in Lactobacillus acidophilus strains having been used for a long time in several fermented milk products. L. acidophilus is one of the predominant lactobacilli in the human intestine and is believed to play an important role in the health and well-being of the gastrointestinal tract. Evidence has suggested that many disorders caused by pathogenic bacteria invading the digestive tract could have been prevented if fermented milk containing proper L. acidophilus strains, which produce several antimicrobial compounds including organic acids, hydrogen peroxide and bacteriocins, had been continuously supplied.1–3 Bacteriocins are proteinaceous bacterial products which have bactericidal activity and are found in various kinds of lactic acid bacteria. Certain L. acidophilus strains that produce bacteriocin have also frequently been found, and a number of bacteriocins including acidocin 8912,4 lactacin B,5 and lactacin F6 were reported.

The L. acidophilus group of lactic acid bacteria have recently been classified into six homologous species of L. acidophilus, L. amylovorus, L. crispatus, L. gallinarum, L. gasseri, and L. johnsonii by a DNA–DNA homology study.7–9 Of these six species, L. gasseri is considered the dominant species inhabiting the human intestine.8–9 Therefore, screening L. gasseri strains is desirable to obtain the best ones for dietary use. No report has so far been presented on the bacteriocin from L. gasseri.

We have found bacteriocin-producing L. gasseri strains in infant faeces,10 and observed that the bacteriocin from L. gasseri had a relatively wide spectrum of bactericidal activity against gram-positive bacteria including enteric pathogens (data to be published). We describe in this report the isolation, purification, and partial characterization of bacteriocin (gassericin A) from L. gasseri.

Materials and Methods

Bacterial strains and media. The bacteriocin producer, Lactobacillus gasseri LA39, had been isolated from human infant faeces as described in the previous report.10 The strain was propagated twice at 37°C in Lactobacilli MRS broth (Difco Laboratories, Detroit, Michigan, U.S.A.) or in modified MRS (DO-MRS) broth. The DO-MRS broth was prepared by dissolving all the ingredients of the MRS broth except Tween 80 (polyoxyethylene(20)sorbitan mono-oleate, Wako Pure Chemical Industries, Tokyo, Japan) in half the volume of distilled water. The solution was then dialyzed by using a Visking cellulose tube of 24Å pore size (Union Carbide Co., U.S.A.; 12–14KDa cut-off) against the same volume of distilled water for 2 days at 4°C in order to remove the high-molecular-weight proteinoeous components. The diffusate was collected, oleic acid (Nacalai Tesque, Kyoto, Japan) was added to attain a final concentration of 0.1% (v/v), and the mixture autoclaved. The bacteriocin indicator, L. delbrueckii subsp. bulgaricus JCM 1002, was obtained from Japan Collection of Microorganisms (JCM, Wako, Japan), the strains being propagated twice as a 1% inoculum through the MRS broth for 16h at 37°C before use.

Bacteriocin assay. Bacteriocin activity was assayed by the agar-well diffusion method.11 Samples mixed with the same volume of a 5 mM sodium phosphate buffer (pH 6.8) or 50 mM sodium phosphate buffer (pH 6.8)/2-propanol (1:1, v/v) were added to each well of an indicator lawn overlaid on MRS agar. After incubating overnight at 37°C under anaerobic conditions with Anaerocult A (E. Merck, Darmstadt, Germany), the bacteriocin titer was determined. One arbitrary unit (AU) is defined as the reciprocal of the highest dilution value showing distinct growth inhibition of the indicator strain.

Production of bacteriocin. The MRS or DO-MRS broth was inoculated at 1% with L. gasseri LA39 and incubated at 37°C. At regular time intervals, a part of the culture was withdrawn and assayed for both its bacteriocin activity and turbidity at 650 nm.

Preparation and purification of bacteriocin

Method A. The MRS broth was used as the growth medium for L. gasseri LA39. After incubating the MRS broth inoculated at 1% with L. gasseri LA39 for 18 h at 37°C, the cells were removed from the culture by centrifugation (5000 × g, 20 min, 4°C). The supernatant was concentrated by ultrafiltration with a model UHP-90 (Advantec Toyo, Tokyo, Japan; 200 kDa MW cut-off) at 10°C. After dialyzing the retentate against a 50 mM Tris–HCl buffer (pH 8.5), the retentate was fractionated by ion-exchange chromatography in a DEAE-Toyopearl 650M column (2.4 × 25 cm; Tosoh Co., Tokyo, Japan) that had been previously equilibrated with the same buffer. Elution was conducted isocratically with the Tris–HCl buffer at 25°C. The active fraction was dialyzed, and then lyophilized. Gassericin A was extracted from the dried sample with 99% methanol, the extract being evaporated under reduced pressure at 35°C and then lyophilized.

Method B. The DO-MRS broth was used as the growth medium for L. gasseri LA39. After incubating the DO-MRS broth inoculated at 1% with L. gasseri LA39 for 36 h at 37°C, the culture supernatant (fraction I) was
obtained by centrifugation (5000 × g, 20 min, 4°C). The supernatant was extensively dialyzed against distilled water at 4°C, concentrated under reduced pressure (fraction II), and then lyophilized. The dried sample was defatted three times with chloroform-methanol (2:1, v/v) at 4°C to remove remaining traces of oleic acid (fraction III). The defatted sample was fractionated by RP chromatography in a LiChroprep RP-8 column (2.6 × 15 cm, E. Merck) by step-wise elution with distilled water, 90% (v/v) acetonitrile, and 90% (v/v) 2-propanol. The fraction eluted with 90% 2-propanol was evaporated under reduced pressure and then lyophilized (fraction IV). After dissolving fraction IV in 60% acetonitrile, the solubilized portion was purified by re-chromatography with the same column by step-wise elution with 20, 40, 60, and 90% 2-propanol. The fraction eluted with 60% 2-propanol was evaporated under reduced pressure, and then lyophilized (fraction V). Gasserin A was dissolved in 60% 2-propanol and stored at −20°C.

**SDS-PAGE and in situ activity assay.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli, using AE-6440 electrophoretic apparatus (ATTO Co., Tokyo, Japan) with a 4.5% concentration of polyacrylamide in the spacer gel and 20% in the separating gel. After an electrophoresis at 20 mA for 2.5 h at 4°C, the gels were stained with Coomassie brilliant blue R-250 (CB, Fluka, Buchs, Switzerland) or with a Silver Stain kit (Wako). MW 2512-16,949 (Pharmacia LKB Biotechnology, Uppsala, Sweden) was used as the molecular weight marker. To find the location of the bacteriocin band in the gel after SDS-PAGE, an in situ activity assay was conducted according to the method of Daba et al.

**Analytical methods.** The protein content of each sample was determined by using Micro BCA protein assay reagent kit (Pierce, Rockford, U.S.A.), with bovine serum albumin (Pierce) as a standard protein.

A sample dissolved in 60% 2-propanol was analyzed by RP-high performance liquid chromatography (HPLC) with a L-6200 pump (Hitachi, Tokyo, Japan), a JASCO UVDEC-100-VI UV detector and DS-L300 data station (Japan Spectroscopic Co., Hachioji, Japan), and a μ Bondapak C18 column (3.9 × 150 mm; Nihon Millipore, Tokyo, Japan). Elution was carried out at 37°C with a linear gradient from 10% to 90% 2-propanol (30 min) at a flow rate of 0.4 ml/min, the effluent being monitored at 220 nm for peptides.

An amino acid composition analysis of the samples was performed according to the method of Bidlingmeyer et al. The samples were hydrolyzed by the gas-phase method with 6 N HCl (Pierce) containing 0.5% phenol (Wako) under vacuum at 108°C for 24 and 72 h. The half-cystine content was determined as cystic acid after performic acid oxidation. Each amino acid was analyzed as a PTC-derivative by RP-HPLC with the same apparatus in a SUPERIOREX ODS S-5μm column (C18, 4.6 × 30 mm; Shiseido Co., Tokyo, Japan). The elution solvents were (A) 10% acetonitrile in a 140 mM sodium acetate buffer at pH 5.4, and (B) 60% acetonitrile in water. Elution was carried out at 40°C with a linear gradient from 100:0 (A/B) to 50:50 (A/B) after 20 min at a flow rate of 1.0 ml/min, the effluent being monitored at 254 nm.

**Results**

**Production of bacteriocin**

As shown in Fig. 1A, the production of gasserin A (640 AU/ml) in the MRS broth reached a maximum in the early stationary phase (12–24 h) and then decreased to a half after 40 h of incubation. As shown in Fig. 1B, the production of gasserin A (120 AU/ml) in the DO-MRS broth reached a maximum in the middle or late stationary phase (24–48 h) and then decreased to a half after 60 h of incubation.
incubation. The optimal incubation times in the MRS and DO-MRS broths were 18 h and 36 h, respectively, for the preparation of gassericin A.

Preparation and purification of bacteriocin

Preparation by Method A. Over 90% of the bacteriocin activity was recovered in the ultrafiltrated retentate, and its specific activity was increased 11-fold with a recovery of 67.4%. No adsorption of the bacteriocin was observed in a DEAE-Toyopearl 650M column equilibrated with a 50 mM Tris–HCl buffer at pH 8.5, nor in a CM-Toyopearl 650M column with a 40 mM sodium citrate buffer at pH 5.0 (data not shown). Extraction of the lyophilized powder with methanol enabled the specific activity of the extract to be increased 90-fold with a 59% recovery. The extracted component migrated as a strange-shaped band by SDS-PAGE, having light blue color after CBB staining (Fig. 2A). This band was like a chicken footprint (A-2) and also carried strong bacteriocin activity according to an in situ activity assay (A-3). A SDS derivative of TWEEN 80 itself migrated as a strange-shaped band having a light blue color after CBB staining (A-1), although it had no bacteriocin activity. The fatty acid analysis by GLC showed that the extract contained a large amount of oleic acid (ca. 5%, w/w) which is considered to have been derived from TWEEN 80, one of the ingredients of the MRS broth (data not shown). Although intensive extraction of the fraction with several organic solvents was attempted, the clear separation of gassericin A from TWEEN 80 was unsuccessful.

Purification by Method B. The results of bacteriocin purification by method B are summarized in Table I. For chromatography with LiChroprep RP-8, high-molecular-weight components were eluted with distilled water, while most of the contaminated peptides were followed by 90% acetonitrile. Bacteriocin activity was found in the eluate of 90% 2-propanol (fraction IV). The bacteriocin adsorbed on LiChroprep RP-18 was not eluted by any other solvents (data not shown). Finally, gassericin A was re-adsorbed on LiChroprep RP-8, and then eluted with 60% 2-propanol (fraction V). The specific activity was increased 4500-fold with a recovery of 6%. Purified gassericin A migrated as a sharp single band by SDS-PAGE with a ca. 3.8 kDa molecular size (Fig. 2B), this being detected by CBB staining, and not by silver staining. Bacteriocin activity was detected overlapping the SDS-PAGE band. Gassericin A showed a single sharp peak at a ca. 60% concentration of 2-propanol on HPLC chromatogram (Fig. 3), but the recovered component after HPLC analysis was missing any bacteriocin activity. By the amino acid composition of fraction V (Table II), and gassericin A was comprised of 45.7% hydrophobic amino acid (Trp, Phe, Leu, Ile, Met, Val, Pro, and Ala). No cysteine nor modified amino acid such as lanthionine or its derivatives were detected in the bacteriocin.

**Table I.** Purification of Gassericin A

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>Total activity (AU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (AU/mg)</th>
<th>Activity recovered (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I (culture supernatant)</td>
<td>2000</td>
<td>246,000</td>
<td>44,000</td>
<td>28</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Fraction II (dialysis retentate)</td>
<td>200</td>
<td>168,000</td>
<td>800</td>
<td>210</td>
<td>68</td>
<td>8</td>
</tr>
<tr>
<td>Fraction III (defatted residue)</td>
<td>100</td>
<td>27,000</td>
<td>700</td>
<td>39</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Fraction IV (RP chromatography)</td>
<td>25</td>
<td>36,000</td>
<td>3.80</td>
<td>4,200</td>
<td>7</td>
<td>150</td>
</tr>
<tr>
<td>Fraction V (re-chromatography)</td>
<td>4</td>
<td>15,000</td>
<td>0.12</td>
<td>125,000</td>
<td>6</td>
<td>4500</td>
</tr>
</tbody>
</table>

* Total activity (AU)/total protein (mg).

Fig. 3. Reverse-phase high-performance Liquid Chromatogram of Gassericin A Prepared by Method B. The purified gassericin A (fraction V) obtained by re-chromatography with LiChroprep RP-8 was loaded into a column of ϕBondasphere 5 p C18, 300 Å, 3.9×150 mm (Millipore) with a linear gradient from 10% to 90% of 2-propanol (30 min). The flow rate was 0.4 ml/min at 37°C with monitoring at 220 nm.

**Table II.** Amino Acid Composition of Gassericin A

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol (%)</th>
<th>No. of residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glx</td>
<td>8.65</td>
<td>3</td>
</tr>
<tr>
<td>Ser</td>
<td>13.18</td>
<td>5</td>
</tr>
<tr>
<td>Gly</td>
<td>17.78</td>
<td>6</td>
</tr>
<tr>
<td>Arg</td>
<td>1.76</td>
<td>1</td>
</tr>
<tr>
<td>Thr</td>
<td>5.69</td>
<td>2</td>
</tr>
<tr>
<td>Ala</td>
<td>14.96</td>
<td>5</td>
</tr>
<tr>
<td>Pro</td>
<td>3.93</td>
<td>1</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.82</td>
<td>1</td>
</tr>
<tr>
<td>Val</td>
<td>2.39</td>
<td>1</td>
</tr>
<tr>
<td>Met</td>
<td>3.33</td>
<td>1</td>
</tr>
<tr>
<td>Ile</td>
<td>3.86</td>
<td>1</td>
</tr>
<tr>
<td>Leu</td>
<td>6.08</td>
<td>2</td>
</tr>
<tr>
<td>Phe</td>
<td>2.39</td>
<td>1</td>
</tr>
<tr>
<td>Trp</td>
<td>10.99</td>
<td>4</td>
</tr>
<tr>
<td>Lys</td>
<td>2.19</td>
<td>1</td>
</tr>
</tbody>
</table>

Amino acid residues were calculated from the molecular weight (3755 Da) estimated by SDS-PAGE.
Discussion

The bacteriocins of lactic acid bacteria can be divided into two groups on the basis of their amino acid composition. One group is low-molecular-weight peptides, "lantibiotics" including nisin,\textsuperscript{16} which have modified amino acids in their molecules.\textsuperscript{17–19} The other group is "anti-lantibiotics," including acidocin 8912,\textsuperscript{20} lactacin F,\textsuperscript{21} lactococcin A,\textsuperscript{22} leucocin A-UAL 187,\textsuperscript{21} pediocin PA-1,\textsuperscript{22} and gassericin A, which are composed of regular amino acids with various sizes of molecules.

The purified gassericin A was slightly soluble in water and became more soluble with increasing addition of alcohol and acetonitrile. The hydrophobic amino acid contents of lactacin F\textsuperscript{23} and acidocin 8912\textsuperscript{4} are 45.6\% and 38.0\%, respectively. The proportion of hydrophobic amino acids in gassericin A was almost the same as that in lactacin F, but it showed more-hydrophobic behavior in the purification step than that of lactacin F. Although most of the bacteriocins could be finally isolated by RP-HPLC in a C\textsubscript{18} column,\textsuperscript{4} gassericin A could only be analyzed in a low-hydrophobic C\textsubscript{8} column because of its strong hydrophobicity.

The production of gassericin A in the MRS broth was two times higher than that in the DO-MRS broth. Although the reason why the bacteriocin activity decreased from the maximum to a lower level during incubation has not yet been fully elucidated, this decrease may have some relationship with hydrolysis by bacterial proteases.

With method A, the presence of Tween 80 in the methanol-extracted active fraction was confirmed by SDS-PAGE, and a high content of oleic acid by GLC analysis. The active area of the bacteriocin on the SDS-PAGE trace overlapped the band of Tween 80. The purified gassericin A obtained by method B was almost insoluble in water, but was soluble in water, alcohol and acetonitrile when Tween 80 was also present. These results strongly suggest that gassericin A was adsorbed with Tween 80 and became a large molecule like a complex polymer, although direct proof such as complex formation is still not apparent. However, this phenomenon provided easy separation of crude gassericin A by alcohol extraction from the MRS broth containing Tween 80.

With method B, the low recovery of total activity after defatting may have been caused by the escape of gassericin A into the solvent. Tween 80 was replaced by oleic acid in the DO-MRS medium. Tween 80 is routinely used as an ingredient in the medium for lactobacilli. We confirmed that oleic acid, a principal constituent of Tween 80, also had a good effect as a growth promoter. The role of the fatty acid in producing or transporting the bacteriocin is still unclear. As \textit{L. gasseri} LA39 did not grow in the MRS broth without Tween 80 (data not shown), oleic acid or its derivative would be essential for cells growth. Although the interference of oleic acid in the purification of gassericin A may be unavoidable, the DO-MRS broth was superior to the MRS broth for producing purified gassericin A.

The new bacteriocin, gassericin A, produced by \textit{L. gasseri} LA39 was an "anti-lantibiotic," being a highly hydrophobic peptide. Further investigations on gassericin A concerning its amino acid sequence and mode of action are in progress. There has recently been the significant report that the activity of bacteriocin from \textit{Lactococcus lactis} LMG 2081 was generated by the complementary action of two peptides.\textsuperscript{23} Increasing research on bacteriocin will provide more information in this field of microbiology.

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References