Gassericin A, a bacteriocin that was produced by *Lactobacillus gasseri* LA39, was treated with l-lysylendopeptidase and 3-bromo-3-methyl-2-(2-nitrophenyl-mercapto)-3H-indole. The fragments were recovered by SDS-PAGE and sequenced. All amino acids of gassericin A were distributed by sequence analysis and the bacteriocin did not contain any modified amino acids. The amino acid sequence of gassericin A completely coincided with that found through the cloning of the structural gene. Gassericin A was shown to be a cyclic bacteriocin (class II) which is bound at the N- and C-terminal ends. This is the first example of a cyclic bacteriocin from lactobacilli lactic acid bacteria.

**Key words:** bacteriocin; *Lactobacillus gasseri*; gassericin A; acidocin B; cyclic structure

Bacteriocins are antimicrobial proteins or protein complexes that act mainly against related bacterial species; those from lactic acid bacteria (LAB) have been classified into four classes (I-IV). These components are important in preventing the growth of harmful bacteria in fermented dairy products. Recently, the threedimensional structure of a class II bacteriocin from LAB, leucocin A, which was produced by *Leuconostoc gelidum* UAL187 was the first of many bacteriocins to be chemically characterized. It may be the most completely characterized bacteriocin from LAB chemically and in regard to mode of action.

The *Lactobacillus acidophilus* group of LAB is widely used in fermented milk products and the intake of these bacteria may have beneficial effects on human health. The *L. acidophilus* group has been classified by DNA sequence similarity and from the composition of the cell wall into six subgroups, including *L. gasseri*. Many *L. acidophilus* strains have been shown to be bacteriocin producers. Gassericin A, a hydrophobic bacteriocin that is produced by *L. gasseri* LA39, isolated from feces from a human infant, has been purified and sequenced by the cloning of its structural gene in our previous study, and its antibacterial spectrum against foodborne pathogenic bacteria has also been described. The *M*ₐ of mature gassericin A was 5,652 by time-of-flight mass spectroscopy. However, the ultimate structure of gassericin A was not clear because N- and C-terminal amino acids were not detected by chemical sequencing. This study describes the complete primary chemical structure of gassericin A, which has an uncommon cyclic structure.

*L. gasseri* LA39 was propagated in Lactobacilli MRS broth (Difco Laboratories, Detroit, MI) or DO-MRS broth (MRS difusate with oleic acid, as replaced by Tween 80). *L. delbrueckii* subsp. *bulgaricus* JCM 1002, an indicator strain for bacteriocin, was obtained from the Japan Collection of Microorganisms (JCM, Riken, Wako, Japan). Both strains were propagated twice in MRS broth with a 1% inoculum at 37°C for 24 h. MRS agar medium was prepared by the addition of 1.5% agar (agar No. 1, Oxoid, Unipath Ltd., Hampshire, UK), and DO soft agar medium was prepared with 0.7% agar.

Bacteriocin activity was assayed by agar well diffusion, as described in an earlier study of ours. Samples were mixed with a 50 mM sodium phosphate buffer (pH 6.8) or a 1:1 (v/v) mixture of 100 mM sodium phosphate buffer and 50% 2-propanol, and were then diluted serially. One unit (U) of activity was arbitrarily defined as the reciprocal of the highest dilution that inhibited the growth of the indicator strain.

The DO-MRS broth was used for the purification of bacteriocin from *L. gasseri* LA 39. Gassericin A in the culture supernatant was isolated as described previously. Purified gassericin A was obtained by repetition of reverse-phase chromatography with a LiChroprep RP-8 resin (E. Merck, Darmstadt, Germany) by stepwise elution with 60% 2-propanol.

SDS-PAGE was done following Laemmli with a 4.5% spacer gel and a 20% separating gel. After electrophoresis at 20 mV for 2 h, half of the gel was stained with Coomassie Brilliant Blue R-250 solution (Fluka Chemie AG, Buchs, Switzerland; 2.5 g/l of a mixture containing 20% methanol and 10% acetic acid). A peptide kit containing markers with *M*, from 2,512 to 16,949 (Pharmacia LKB Biotechnology, Uppsala, Sweden) was used. To detect the activity of bacteriocin on the gel, the other half of the gel was used for an *in situ* assay by the method of Daba et al.

After the purified gassericin A was blotted onto a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore Corp., Bedford, MA), a 75% (v/v) acetic acid solution (100 µl) containing 100 µg of 3-bromo-3-methyl-2-(2-nitrophenyl-mercapto)-3H-indole (MMN-
indole, Fluka) was added to cleave the tryptophanyl bonds in the bacteriocin, and then it was incubated at 47°C for 1 h in darkness. The peptide fragments in the reaction solution and on the membrane were recovered, separated by SDS-PAGE, and then extracted from gels with 70% (v/v) formic acid. This was followed by an amino acid sequence analysis with an automatic protein sequencer (model 473A), an on-line phenylthiohydantoin analyzer (model 120A), and a data analysis system (model 610A; Applied Biosystems, Inc., Foster City, CA). The gassericin A that was digested by lysylendopeptidase (EC 3.4.21.50) on the poly(vinylidene difluoride) membrane was sequenced directly.

Figure 1 shows the results of an SDS-PAGE of purified gassericin A and digests by MMNM-indole. Purified gassericin A (M, 5,652 by mass spectroscopy) migrated with a molecular weight of 3,800 as a sharp single band, as is shown in lane 2. After digestion by MMNM-indole, gassericin A was cleaved into 3 bands, A, B, and C. They were recovered from the gel and their amino acids were sequenced.

Band B had its first 25 amino acid residues sequenced (IADQFGIHATGARKLLDAMASGA), including the cleavage site via lysylendopeptidase. Band A was a peptide composed of 15 amino acids (ALAAAGAL-GATAAIY) including C- (Ala) and N-terminal amino acid (Ile) moiety in the preceding structure which was observed through the cloning of the structural gene of gassericin A. The presence of a bond between isoleucine and alanine showed that gassericin A is a cyclic bacteriocin that is linked at N- and C-terminals.

Hydrolysis of gassericin A with lysylendopeptidase made it possible to first sequence 27 amino acid residues that contained a tryptophan residue that was located at the C-terminal side of the gassericin A (LDMASGAS- LGTAFACAILGVTPAWA). We noted in a previous study that the gassericin A digested by lysylendopeptidase migrated as a larger molecular component in a band of M, 5,600 on SDS-PAGE, suggesting that gassericin A changed from a cyclic structure into a rod form through enzymatic cleavage at the sole lysyl residue in its structure.

Only the first tryptophan residue, at the N-terminal side of gassericin A, remained undetected since the tryptophan residue was modified by the treatment with MMNM-indole. Band C (M, 5,600) was considered to be a mixture of two rod form fragments from disruption at each tryptophan residue by MMNM-indole (Fig. 2). The sequence of band C in Fig. 2 was estimated by amino acid sequencing. The two amino acid peaks appeared on every chromatogram and the tryptophan peak was observed on the 16th chromatogram together with a lysine peak.

Because two tryptophan residues were found in the predicted sequence of gassericin A through the cloning of the structural gene, Fig. 2 shows the circular whole amino acid sequence of gassericin A together with the sequences of the fragments after treatment with MMNM-indole and lysylendopeptidase. The amino acid sequence of gassericin A was the same as the predicted one through the cloning of the structural gene. Finally, all the amino acid sequences of gassericin A were chemically discovered. The bacteriocin did not contain any modified amino acids such as lanthionine. Gassericin A (class II) is a cyclic bacteriocin that is bound at N- and C-terminal ends. This is the first data on bacteriocins from Lactobacillus species. The bacteriocins from Enterococcus faecalis strains 18,19 are other instances of cyclic form.

Acidocin B, which is produced by L. acidophilus M46,20 has high sequence similarity (98%) with gassericin A. The molecular weight of acidocin B was measured as 2,400 on SDS-PAGE in spite of the molecular weight being calculated to be 5,800 from the DNA se-
quence and amino acid analysis. The behavior of these bacteriocins on SDS-PAGE was related to the tertiary structure and acidocin B might be a cyclic bacteriocin like gassericin A.

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