Identification of the Lantibiotic Nisin Q, a New Natural Nisin Variant Produced by Lactococcus lactis 61-14 Isolated from a River in Japan

Takeshi ZENDO,1 Masanori FUKAO,1 Kyoko UEDA,2 Tomoko HIGUCHI,2 Jiro NAKAYAMA,1 and Kenji SONOMOTO1,

1Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
2Biotechnology & Food Research Institute, Fukuoka Industrial Technology Center, 1465-1 Aikawacho, Kurume, Fukuoka 839-0861, Japan

Received January 17, 2003; Accepted April 25, 2003

Lactococcus lactis 61-14 isolated from river water produced a bacteriocin active against a wide range of Gram-positive bacteria. N-terminal amino acid sequencing, mass spectral analysis of the purified bacteriocin, and genetic analysis using nisin-specific primers showed that the bacteriocin was a new natural nisin variant, termed nisin Q. Nisin Q and nisin A differ in four amino acids in the mature peptide and two in the leader sequence.

Key words: nisin; bacteriocin; lantibiotic; lactic acid bacteria; Lactococcus lactis

Bacteriocins are bacterial peptides or proteins with antibacterial activity against bacterial strains closely related to producing strains. Bacteriocins produced by lactic acid bacteria (LAB) have attracted special interest in the aspect of their potential use as safe food preservatives. The most extensively studied LAB bacteriocin is nisin, produced by Lactococcus lactis strains. Nisin, which was first discovered in 1928,1) contains unusual amino acid residues, namely dehydroalanine, dehydrobutyrine, lanthionine, and 3-methylanthionine. Bacteriocins containing these unusual amino acids are classified as lantibiotics.2,3) In lantibiotics, unusual amino acids might be formed by the following enzymatic post-translational modification (dehydration and intra-thioether linkage).4,5) First, dehydration of serin and threonine forms the unsaturated amino acids, dehydroalanine and dehydrobutyrine (DHB), respectively. Subsequently, some of the DHA and DHB residues were covalently linked to a free thiol group of a cysteine within the same peptide to form lanthionine and 3-methyl-lanthionine, respectively.

Until now, two natural nisin variants, nisin A and nisin Z, have been recognized. Structures of nisins A and Z were determined in 1971 and 1991, respectively.5,7) Nisins A and Z differ in one amino acid residue at position 27 as a consequence of a single nucleotide substitution. Nisin A contains histidine, and nisin Z asparagine. This provides nisin Z with higher solubility at neutral pH than nisin A.8) Nisins A and Z have bactericidal activity against a wide range of Gram-positive bacteria, including food spoilage bacteria and foodborne pathogens. Nisin A was approved as a safe food preservative by the World Health Organization (WHO) and has been used as a practical food preservative in more than 50 countries.9) In this study, we described identification of a new natural nisin variant, nisin Q, produced by a newly isolated lactic acid bacterium.

Strain 61-14, isolated from river water collected in the Hikosan River (Tagawa, Fukuoka, Japan), showed bacteriocin-like activity. Strain 61-14 was identified as L. lactis by sugar fermentation tests using an API 50 CHL identification kit (bioMérieux, Marcy l’Etoile, France) and sequencing analysis of 16S rRNA gene. Antibacterial activity in the culture supernatant of L. lactis 61-14 was assayed by the spot-on-lawn method.10) The culture supernatant inhibited the growth of a wide range of Gram-positive bacteria including LAB, Bacillus sp., Listeria sp., and Micrococcus sp. The antibacterial spectrum was similar to that of nisin (data not shown). However, the spectrum was not completely identical to those of nisins A and Z. The antibacterial activity was completely inactivated by proteolytic enzymes, proteinase K and actinase E. This supported the idea that the antibacterial activity was derived from a proteinaceous substance, bacteriocin.

Purification of the bacteriocin produced by L. lactis 61-14 was done by the procedure described by Cintas et al.11) with some modifications. L. lactis

† To whom correspondence should be addressed. Fax: +81-92-642-3019; E-mail: sonomoto@agr.kyushu-u.ac.jp
61-14 was grown in a 1-liter MRS medium (Oxoid, Hampshire, England) at 30°C until early stationary phase. The cell-free supernatant was shaken with 20 g of Amberlite XAD-16 (Sigma, St. Louis, MO) for 3 h at room temperature. The matrix was then packed into a column and washed with 100 ml of distilled water and 100 ml of 40% ethanol. The bacteriocin was eluted with 70% 2-propanol containing 0.1% trifluoroacetic acid (TFA). The eluate was put on at a flow rate of about 2 ml/min to an SP-Sepharose Fast Flow cation-exchange column (Amersham Pharmacia Biotech, Tokyo; 100 mm length, 15 mm internal diameter) equilibrated with 20 mM sodium phosphate buffer, pH 5.7 (buffer A). The elution was done with a stepwise gradient from 0 to 1 M NaCl in buffer A. The active fraction obtained (ca. 0.5 M NaCl) was then put on to a C18 reverse-phase column (PepRPC HR 5/5, Amersham Pharmacia Biotech) integrated in LC-10A high performance liquid chromatography system (Shimadzu, Kyoto, Japan). The integrated column was then packed on to a C2 reverse-phase column (Amersham Pharmacia Biotech) and the elution was done with a stepwise gradient ranging from 0 to 100% 2-propanol in aqueous 0.1% TFA for 30 min at a flow rate of 1.0 ml/min.

The N-terminal amino acid sequencing analysis of the purified bacteriocin was done by Edman degradation using a PSQ-1 gas-phase automatic sequence analyzer (Shimadzu). Only the first N-terminal amino acid was identified as isoleucine, and the subsequent amino acids were not detected. Without any reducing treatments, sequencing analyses of purified nisins A and Z peptides would lead to the same result, because they have isoleucine at position 1, dehydrobutyrine at position 2, and lanthionine at position 3. This implied the existence of an unusual amino acid at positions 2 and/or 3 of the bacteriocin from L. lactis 61-14 in the same manner as nisins A and Z.

Meyer et al. have reported that a treatment with alkaline 2-mercaptopethanol cleaves the thioether bonds of lanthionine and 3-methyl-lanthionine and allows Edman degradation of lantibiotics. Using this procedure on the bacteriocin gave an N-terminal amino acid sequencing analysis using a Voyager DE-STR mass spectrometer (PE Biosystems, Foster City, CA). The molecular mass of the purified bacteriocin was 3,327.31 Da. It was slightly less than those of nisins A and Z measured by the same procedure (3,354.53 Da and 3,330.91 Da, respectively).

To compare the bacteriocin from L. lactis 61-14 with nisin in amino acid sequence, the DNA sequence encoding the bacteriocin was analyzed. The whole genome of L. lactis 61-14 was isolated using a MagExtractor Genome (Toyobo, Osaka, Japan) and used as a DNA template for PCR amplification. Primers designed from the 5' and 3' flanking sequences of the nisin Z structural gene7) were used to obtain the gene encoding the nisin-like bacteriocin from strain 61-14. PCR amplification was done using Premix Taq DNA polymerase (Ex Taq Version, Takara Bio, Otsu, Japan) under conditions consisting of 30 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec, and polymerization at 72°C for 1 min. PCR presented a single product the size of which was identical to the expected one of the nisin structural gene. The amplified product was cloned in Escherichia coli JM109 using pUC18, and sequenced. The sequence was confirmed by sequencing of multiple dependent PCR products. The sequence presented in this article has been submitted to GenBank and assigned the accession number (AB100029).

The DNA sequencing of the cloned gene, designated nisQ, showed that it encoded a peptide containing a determined amino acid sequence with high similarity to nisins A and Z (Figs. 1 and 2). The deduced bacteriocin comprised 57 amino acid residues in the prepeptide and 34 residues in the mature peptide, as nisins A and Z did. The structure of the mature pep-
tide was proposed, based on the above-mentioned N-terminal amino acid sequence of the chemically modified peptide and post-translational modification and cleavage of the leader peptide similar to nisins A and Z (Fig. 3). The molecular mass was calculated to be 3,327 Da, which corresponded to the observed one of 3,327.31 Da by MALDI-TOF MS. Thus, the bacteriocin from *L. lactis* 61-14 was a novel natural nisin variant. We termed the bacteriocin nisin Q as a new natural nisin variant.

An alignment of amino acid sequences of nisin prepeptides is shown in Fig. 2. Six amino acids were different between nisin A and nisin Q prepeptides, while only one amino acid between nisins A and Z. In mature peptides after the post-translational modification, there were four amino acid substitutions between nisins A and Q, and three between nisins Z and Q. The nisin A and Z molecules consist of N-terminal and C-terminal domains connected by methionine at position 21, acting as a flexible hinge region. In nisin Q the methionine was replaced by leucine as in subtilin, one of the nisin-type lantibiotics. Additionally, nisin Q, nisin Z, and subtilin contain asparagine at position 27, while nisin A contains histidine. The structure-function relationship of nisin has been widely studied by site-directed mutagenesis. Some studies showed that dehydrated residues and lanthionine or 3-methyllanthionine rings in nisin were important for the activity. Nisin Q conserved a structure composed of such unusual amino acids, which probably provides nisin Q with a broad antibacterial spectrum like other nisin variants. The influence of amino acid substitution in the leader peptide was also studied by site-directed mutagenesis. It showed that the conserved residues at positions from −18 to −15 in nisin-type lantibiotics were essential for biosynthesis. On the other hand, the proline residue at position −2 was not important, although it was highly conserved. These results agreed well with the leader peptide sequence of nisin Q, which conserved the residues at positions from −18 to −15 but not the residue at position −2 (Fig. 2).

Further work is needed for detailed characterization and complete structural analysis of nisin Q. Comparison in characteristics of nisin Q with nisins A and Z must give valuable information about the structure-function relationships of lantibiotics, which have not been solved entirely yet.

### Acknowledgment

We are grateful to M. Kimura and Y. Kouzuma (Kyushu University, Fukuoka, Japan) for amino acid sequencing and to K. Ogawa (Asahi Kasei Corporation, Ohito, Japan) for amino acid composition analysis.

### References

10. Ennahar, S., Asou, Y., Zendo, T., Sonomoto, K., and Ishizaki, A., Biochemical and genetic evidence for production of enterocins A and B by *Enterococ-
Nisin Q, a New Natural Nisin Variant


