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https://doi.org/10.5109/24265

出版情報：九州大学大学院農学研究院紀要. 43 (1/2), pp.195-207, 1998-11. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **43** (1·2), 195–207 (1998)

Pediococcus sp. ISK–1 Produces a Novel Lantibiotic, Bacteriocin ISK–1

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(Received June 19, 1998 and accepted August 7, 1998)

Bacteriocin ISK–1 is a proteinaceous inhibitory substance produced by *Pediococcus* sp. ISK–1 isolated from well–aged *Nukadoko* (Kimura *et al.*, 1997a). Bacteriocin ISK–1 was purified by acid treatment, ammonium sulfate precipitation, cation–exchange chromatography and reversed–phase high performance chromatography from the culture supernatant of *Pediococcus* sp. ISK–1. Purification of bacteriocin ISK–1 resulted in 30–fold increase in the specific activity and the recovery was 17%. Molecular mass of bacteriocin ISK–1 determined by fast atom bombardment–mass spectrometry was 2,960. The amino acid composition analysis of bacteriocin ISK–1 revealed that it contained lanthionine and/or 3–methyllanthionine which is a characteristic of lantibiotics. The N–terminal amino acid sequence analysis indicated the first seven N–terminal amino acid residues as, NH_2–K–K–K–S–G–V–I. The primary sequence showed significant homology to the lantibiotics lacticin 481 from *Lactococcus lactis* and variacin from *Micrococcus varians*, which suggests that bacteriocin ISK–1 is a novel lantibiotic belonging to a lacticin–481 type.

INTRODUCTION

Many researches for lactic acid bacteria are interested because of their contribution to food industry such as flavor and antimicrobial substances. Bacteriocins of lactic acid bacteria are proteinaceous substances, some of which bactericidally or bacteriostatically inhibit the growths of food–born or pathogen bacteria such as *Listeria* and *Clostridium*. They are generally stable over a wide range of pH and can be degraded by various kinds of proteinases, which make them potential as natural food preservatives in the near furture (Tagg *et al.*, 1976; Klaenhammer, 1988; Jack *et al.*, 1995). Among four classes of bacteriocins (Klaenhammer *et al.*, 1993), lantibiotics, class I bacteriocins, are currently attracting considerable attention because of their biosynthesis and their wide range of antimicrobial spectra.

Lantibiotics are defined as lanthionine– and/or 3–methyllanthionine–containing peptides with antimicrobial activity (Sahl *et al.*, 1995). They generally contain unsaturated amino acids such as dehydroalanine and dehydrobutyrine. Lanthionine and 3–methyllanthionine are formed by dehydration of a serine and a threonine to form dehydroalanine and dehydrobutyrine, respectively, followed by addition of the thiol group of a cysteine to the unsaturated amino acid residue (Klaenhammer, 1993; Sahl *et al.*, 1995). Lanthionine and dehydroamino acid have been suggested to confer stability to the active conformation of the lantibiotic against heat and acids (Kuipers *et al.*, 1992) and

against proteinases present in the producer cells (Bierbaum *et al.*, 1995). Biosynthesis of lantibiotics includes ribosomal synthesis, posttranslational modification reactions, transport across the cell membrane and cleavage of the leader peptide (Klaenhammer, 1993; Sahl *et al.*, 1995). According to the biosynthetic peculiarities, structural properties and biological activities, lantibiotics can be classified into two types (Jung, 1991). Type A comprises more elongated screw-shaped, amphiphilic polypeptides and exerts their primary bactericidal action by the formation of pores and depolarization of the bacterial cytoplasmic membrane. Type B consists of more compact, globular, and almost neutral amphiphilic polypeptides and appears to act by the inhibition of enzymatic activities (Jung, 1991). Furthermore, type A lantibiotics can be distinct into two subgroups in respect of both their ring structure and their leader peptide with a characteristic cleavage site (Havarstein *et al.*, 1994; Hynes *et al.*, 1994; Sahl *et al.*, 1995; Pridmore *et al.*, 1996; van den Hooven *et al.*, 1996). Nisin-type subgroup includes nisin (Gross and Morell, 1971; Mulders *et al.*, 1991; Matsusaki *et al.*, 1996), subtilin (Chan *et al.*, 1993), epidermin (Allgaier *et al.*, 1986), Pep5 (Kellner *et al.*, 1989), and epilancin K7 (van den Kamp *et al.*, 1995), gallidermin (Kellner *et al.*, 1988), mutacin B-Ny266 (Mota-Meria *et al.*, 1997), whereas lacticin 481-type subgroup contains lacticin 481 (Piard *et al.*, 1992; van den Hooven *et al.*, 1996), streptococcin A-FF22 (Jack *et al.*, 1994), salivaricin A (Ross *et al.*, 1993), streptococcin A-M49 (Hynes *et al.*, 1994), and variacin (Pridmore *et al.*, 1996). Although lactocin S (Mørtvedt *et al.*, 1991) and cytolysin (Gilmore *et al.*, 1994) have leader peptides showing some similarities with those of lactacin 481-type subgroup (Havarstein *et al.*, 1994), unrelated propeptide parts were to be classified in a third subgroup (Sahl *et al.*, 1995).

Nisin, produced by some strains of *Lactococcus lactis* was the first type A of lantibiotic to be characterized genetically and biochemically in details (Gross and Morell, 1971; Buchman *et al.*, 1988; Kaletta and Entian, 1989; Engelke *et al.*, 1992, 1994; Kuipers *et al.*, 1993, 1995; van der Meer *et al.*, 1993). Nisin has two natural variants, nisin A (Gross and Morell, 1971) and nisin Z (Mulders *et al.*, 1991; Matsusaki *et al.*, 1996). Nisin A and nisin Z differ by a single amino acid substitution at position 27, with His in nisin A and Asn in nisin Z (Mulders *et al.*, 1991; Matsusaki *et al.*, 1996). Nisin is synthesized ribosomally as a precursor peptide of 57 amino acids. It undergoes posttranslational modifications, including cleavage of a leader peptide of 23 amino acids, and mature nisin is secreted (Gross and Morell., 1971; Buchman *et al.*, 1988; Kaletta and Entian, 1989). Nisin inhibits not only the growth of a wide range of Gram positive bacteria but also the germination and/or outgrowth of spores of *Bacillus* and *Clostridium* species (Hurst *et al.*, 1981). Therefore, nisin has been widely used as a food preservative (Delves-Broughton *et al.*, 1990). However, the use of food industries has so far been limited because of its insolubility at a neutral pH. Although protein engineering can make nisin Z soluble at neutral pH (Rollema *et al.*, 1995), consumer can not accept the use of engineered bacteriocin as food preservatives yet. Therefore, it is necessary to find novel natural bacteriocins with enough antimicrobial activity to be used safety as food preservatives.

Salt-tolerant *Pediococcus* sp. ISK-1 isolated from well-aged *Nukadoko*, the bed of fermented rice bran, was found to produce a novel bacteriocin, bacteriocin ISK-1 (Kimura *et al.*, 1997b). Bacteriocin ISK-1 might play an important role in contributing to maintain

the stable microflora in *Nukadoko*. Since the bacteriocin was stable over a wide range of pH and was heat resistant under acidic conditions, it is possible to use as a food preservative (Kimura *et al.*, 1997b). In this study, the purification and the identification of bacteriocin ISK-1 were carried out. The results mentioned here showed that bacteriocin ISK-1 was a novel lantibiotic.

MATERIALS AND METHODS

Strains and culture condition

Pediococcus sp. ISK-1, a new strain isolated in our laboratory from well-aged fermented *Nukadoko*, was used in this study. It was proved that mevalonic acid markedly stimulates the lactate fermentation by ISK-1 strain, that is, increasing the growth, substrate consumption, and product formation rates (Herawati and Ishizaki., 1997, 1998). *Pediococcus acidilactici* JCM 5885^T was used as an indicator microorganism for assay of antimicrobial activity. *P. acidilactici* was grown in thioglycolate medium without glucose (TGC medium; Difco Laboratories, U. S. A.) at 37 °C for 18 h.

For the production of bacteriocin ISK-1, the stock culture of *Pediococcus* sp. ISK-1 was statically subcultured in 10 ml of TGC medium at 37 °C for 18 h and then transferred to 100 ml of MRS medium (Oxoid, England) at pH 7.0 for preculture. An aliquot of 25 ml of the preculture, after growth at 37 °C for 3 h, was inoculated into a 1-liter jar fermentor with a working volume of 500 ml of MRS medium. The fermentation was carried out at pH 6.0, maintained by a feeding system that supplied 3 N NaOH, at 37 °C with agitation speed of 440 rpm without aeration.

Analytical condition

Cell density was monitored by absorbance at 562 nm and converted to dry cell weight from a standard curve. Glucose concentration was analyzed according to the method described previously (Ishizaki *et al.*, 1992).

Bioassay

The antimicrobial activity was detected by the agar disc diffusion assay. The assay was performed as follows. One hundred microliters of 100-fold diluted indicator culture grown for 18 h was seeded in 10 ml of MRS medium containing 0.7% agar. The mixture was poured into Petri dishes. After solidification of the soft agar media, sterile paper discs (8 mm diameter) with 40 μl of either original or a serial 2-fold diluted sample of bacteriocin ISK-1 were placed on the surface. After the plates were incubated at 37 °C for 18 h, inhibitory zones formed around the paper discs were measured for the quantitative analysis. The relationship between the diameter (R) of the inhibitory zones and the amount of bacteriocin sample sank into a paper disc (d) can be represented by the following equation: $R=a+b\log(d)$, $d=40/(1000\times D)$, where D indicates a dilution rate of a bacteriocin sample. The critical dose (CD) was defined as the amount of bacteriocin solution corresponding to a null inhibition zone and calculated by extrapolating the equation. The titer, in AU/ml, was calculated as the reciprocal of the CD. The standard curve, relating dose in AU to R, had a zero intercept and the same slope of the above-mentioned equation. The activity in AU/ml of a sample was calculated as follows:

AU/ml=(1000/40)×D×$10^{(R/6)}$ (Parente *et al.*, 1995).

Purification

The culture supernatant of *Pediococcus* sp. ISK-1 was adjusted to pH 3.0 with concentrated HCl and was allowed to stand overnight at 4°C. After the precipitate formed was removed by centrifugation at 16,000×g for 20 min, the active supernatant was brought to 70% saturation by the addition of solid ammonium sulfate and allowed to stand overnight at 4°C. The precipitate was collected by centrifugation at 16,000×g for 30 min and then suspended in a small amount of 0.01 N HCl. This suspension was dialyzed against 6 M urea-HCl (pH 3.0) in a Spectra/Por membrane (Spectrum Medical Industries, Inc., USA; molecular weight cut off, 1,000) to desalt the protein and disrupt some aggregates. Further purification of the bacteriocin was carried out by cation-exchange chromatography on a column of CM-Shephadex C-25 (Pharmacia, Sweden; 5 cm i.d.×20 cm). The antimicrobially active dialyzate was loaded on the column and washed with 50 mM acetate buffer (pH 3.8) at a flow rate of 0.8 ml/min. The column was subsequently eluted with a 0-1.5 M NaCl-50 mM acetate buffer (pH 3.8), as a stepwise elution, at a flow rate of 0.8 ml/min. Absorbance was monitored at 280 nm. The antimicrobially active fractions obtained from cation-exchange chromatography were loaded on a tC_{18} Sep-Pak cartridge (Waters, U. S. A.), and the column was washed with 0.05% trifluoroacetic acid (TFA). The fraction with antimicrobial activity was eluted with 50% acetonitrile in 0.05% TFA to be desalted and concentrated. The eluate was then dried by Speed Vak Concentrator (Savant Instruments, Inc.,U. S. A.). The dried material was dissolved in a small amount of 0.01 N HCl. Further purification was performed by reversed-phase high performance liquid chromatography (HPLC) on an Asahipak ODP-50 column (Showa Denko Co., Ltd., Japan). For the mobile phase, solvent A was 0.05% TFA in distilled water and solvent B was 0.05% TFA in 100% acetonitrile. For preparative fractionation, a large size of column (21.5 mm i.d.×300 mm) was used with a linear gradient of 25-35% solvent B in solvent A over the course of 50 min at a flow rate of 4 ml/min. For analytical chromatography, a small size of column (6.0 mm i.d.×250 mm) was also used with a linear gradient of 25-35% solvent B in solvent A over the course of 50 min at a flow rate of 1 ml/min. Absorbance was monitored at 210 nm. The active peak fraction was collected and lyophilized.

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was performed by the method of Schägger & von Jagow (1987) in 16.5% T-6% C gels. T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide). C denotes the percentage concentration of the cross-linker relative to the total concentration T. A molecular mass marker kit (MW-SDS-17; Sigma Chemical Company, U. S. A.) was used as a source of standard protein. After electrophoresis, the gel was stained with 0.025% SERVA blue G (Serva, Germany) in 10% acetic acid.

Molecular mass determination

The molecular mass of bacteriocin ISK-1 was determined by fast atom bombardment-mass spectrometry (FAB-MS) with a mass spectrometer (JMS-SX102; JEOL, Japan). Glycerol and thioglycerol were used as the matrix.

Amino acid composition and sequence analysis

The purified bacteriocin ISK-1 was hydrolyzed in 6 N HCl under a vacuum at 110°C for 24 h. The amino acid analysis of the hydrolyzate was carried out with a Hitachi L-8500 amino acid analyzer (Hitachi, Japan).

The N-terminal amino acid sequence of bacteriocin ISK-1 was analyzed by Edman degradation with an automated gas-phase protein sequencer (PSQ-1; Shimadzu, Japan) with an on-line LC-6A HPLC system (Shimadzu).

RESULTS

Production of bacteriocin ISK-1

Figure 1 shows the time course of the production of bacteriocin ISK-1 during the growth of *Pediococcus* sp. ISK-1. The activity of the culture supernatant was determined by bioassay after being desalted and concentrated by a tC_{18} Sep-Pak cartridge and Speed Vak Concentrator to remove inhibitory substances such as organic acids.

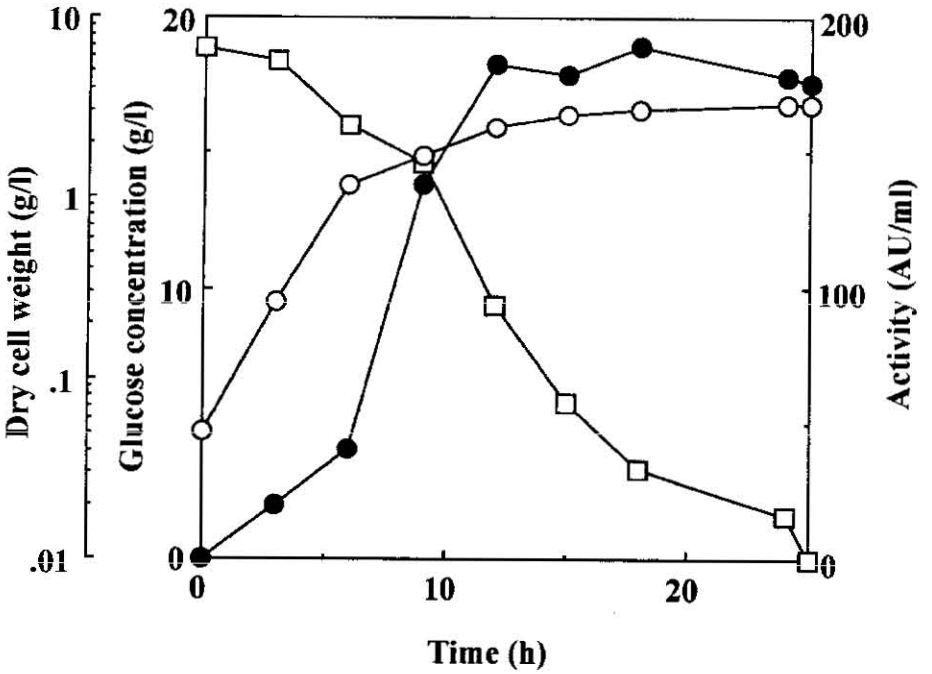

Fig. 1. pH-controlled batch fermentation profile of *Pediococcus* sp. ISK-1.
Symbols: ○, cell growth; □, residual glucose concentration; ●, activity of bacteriocin ISK-1.

Table 1. Purification of bacteriocin ISK-1.

Sample	Volume (ml)	Total protein[a] (mg)	Total activity ($\times 10^3$ AU)	Specific activity ($\times 10^3$ AU/mg)	Recovery (%)	Purification (-fold)
Culture supernatant after acid treatment (pH 3.0)	500	352	90.5	0.257	100	1
Ammonium sulfate precipitate (70% saturation)	20	40.0	95.0	2.38	105	9.30
Cation-exchange extract	240	9.50	42.8	4.51	47.3	17.5
C_{18}-HPLC extract	10	2.12	15.1	7.12	16.7	27.7

[a]Protein was estimated by the method of Lowry *et al.* (1951).

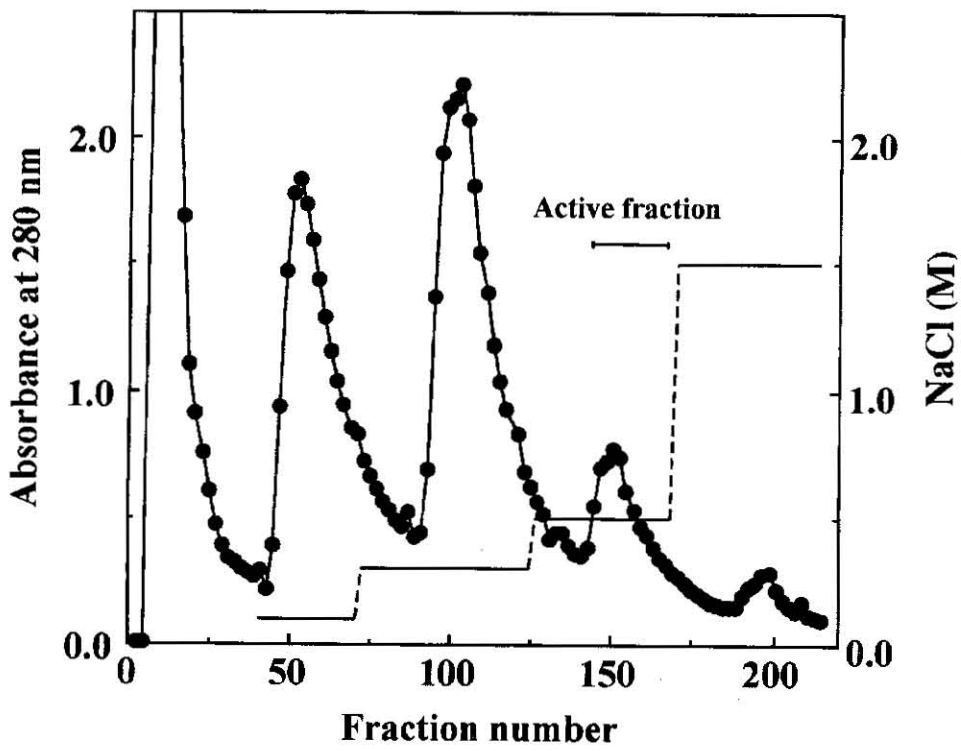

Fig. 2. Purification of bacteriocin ISK-1 by CM-Sephadex C-25.
Symbols: ●, absorbance at 280 nm; —, NaCl concentration.
Fractions 130-159, indicated by the bar, were collected for further purification.

Bacteriocin ISK-1 was produced in proportion to the extent of cell growth and reached the maximum level of 189 AU/ml at the late of stationary phase of growth. The culture supernatant at the phase was collected for the subsequent purification of bacteriocin ISK-1.

Purification of bacteriocin ISK-1

Bacteriocin ISK-1 was purified by acid treatment, ammonium sulfate precipitation, and sequential cation-exchange chromatography and reversed-phase HPLC (Table 1).

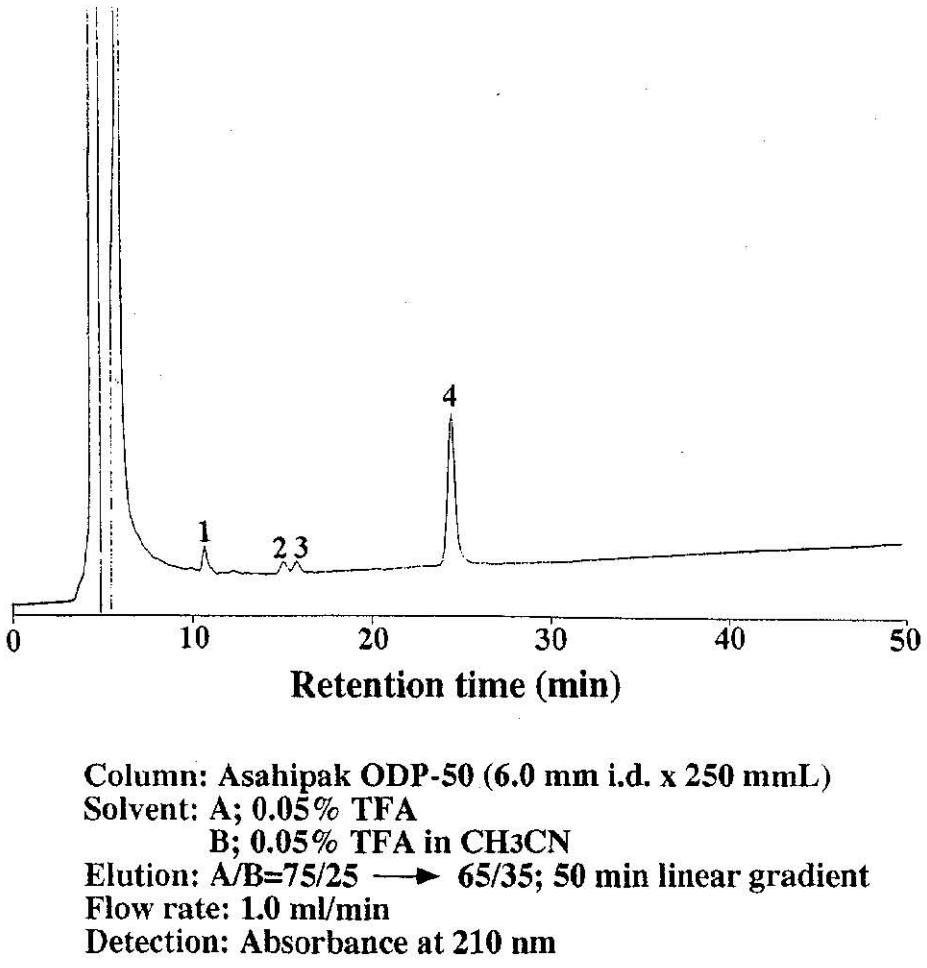

Fig. 3. Analysis by reversed-phase HPLC of the crude bacteriocin ISK-1. Bacteriocin ISK-1 obtained from CM-Sephadex C-25 was subjected to HPLC.

Ammonium sulfate precipitation resulted in a ten-fold increase in the specific activity and 105% of recovery of the activity was obtained. After dialysis, the active material was loaded on CM-Sephadex C-25 column. Fractions 130-159 with antimicrobial activity were eluted with 0.5 M NaCl (Fig. 2). The specific activity increased 20-fold and recovery of the activity was about 47%. The active fractions were then analyzed by reversed-phase HPLC. As shown in Fig. 3, the elution profile revealed four peaks. Although antimicrobial activities were detected in peak fractions, nos. 2, 3, and 4, more than 80% of the activity in the total eluate was detected in peak fraction, no. 4. The other peak fractions might be a sort of degraded bacteriocin ISK-1. Consequently, about 30-fold increase in specific activity and total recovery of 17% were obtained.

The purity of bacteriocin ISK-1 was assessed by Tricine-SDS-PAGE. After peak fraction no. 4 was concentrated and dried with tC_{18} Sep-Pak cartridge and Speed Vak Concentrator, bacteriocin ISK-1 was dissolved in a small amount of 0.01 N HCl. The

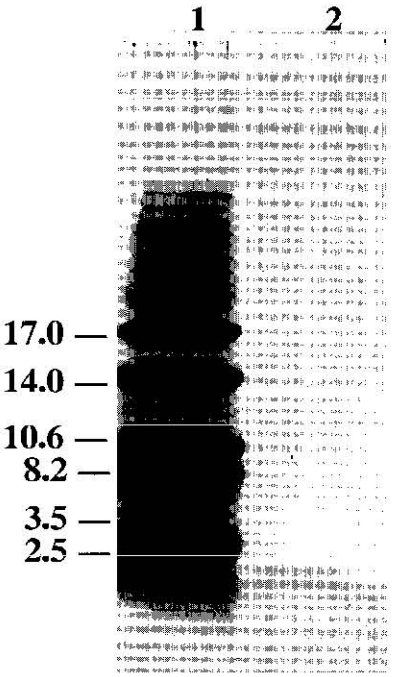

Fig. 4. Tricine-SDS-PAGE of the purified bacteriocin ISK-1. Lanes: 1, low-molecular-mass standard (kDa): 2, peak fraction no. 4 obtained by HPLC.

sample was subjected to Tricine-SDS-PAGE. A single band was detected, which indicated that the bacteriocin was purified to homogeneity by the purification method described above. In the gel electrophoresis, bacteriocin ISK-1 migrated with an apparent molecular mass of about 3 kDa (Fig. 4).

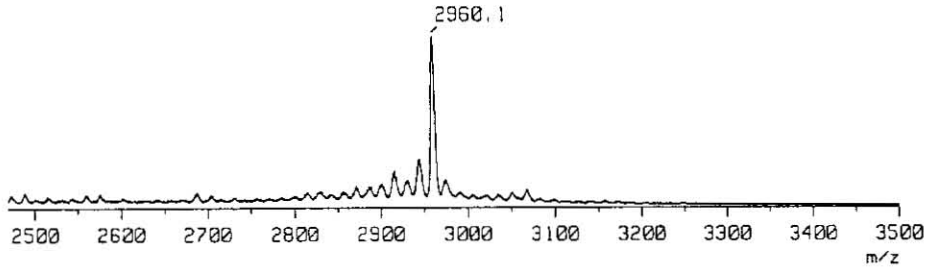

Fig. 5. FAB-MS spectrum of bacteriocin ISK-1.

Molecular mass determination by mass spectrometry

Molecular mass determination of the purified bacteriocin ISK-1 was carried out by FAB-MS (Fig. 5). The molecular mass of bacteriocin ISK-1 was 2,960 under the measurement conditions described in Materials and Methods.

Table 2. Amino acid composition of bacteriocin ISK-1.

Residue	Bacteriocin ISK-1 (mol/mol)
Asx	1.9
Ser	1.4
Glx	1.0
Gly	1.2
Val	2.4
Met	0.8
Ile	0.8
Phe	2.7
Lys	2.7
His	1.8
Pro	1.1
Lan	3.2

Lan denotes DL-lanthionine and represents an estimate of the amount of lanthionine plus 3-methyllanthionine content.

Table 3. Alignment of the N–terminal amino acid sequences of type A lantibiotic prepeptides.

Lantibiotic	N–terminal sequence
Lacticin–481 type	
Bacteriocin ISK–1	K K K S G V I
Lacticin 481	K G G S G V I
Variacin	– – G S G V I
Streptococcin A–FF22	– G K N G V F
Salivaricin A	K R G S G W I
Nisin–type	
Nisin A, Z	I T S I S L C
Subtilin	W K S E S L C
Epidermin	I A S K F I C
Pep5	T A G P A I R
Epilancin K7	S A S V L K T
Gallidermin	I A S K F L C
Mutacin B–Ny266	F K S W S F S

Identical residues are indicated by boxes.

Amino acid composition and sequence

Amino acid composition of bacteriocin ISK–1 was analyzed (Table 2). Lanthionine and/or 3–methyllanthionine were detected using DL–lanthionine as a standard. This indicates that bacteriocin ISK–1 could be a lantibiotic. The N–terminal amino acid analysis revealed the following sequence: NH_2–K–K–K–S–G–V–I. However, Edman degradation was inhibited at the eighth amino acid residue. This suggests that a lanthionine or a dehydroamino acid may exist at the position such as nisin (Jung, 1991; Matsusaki *et al.*, 1996) and lacticin 481 (van den Hooven *et al.*, 1996) which are lantibiotics. Table 3 shows the alignment of N–terminal amino acid sequences of type A lantibiotics. The sequence of bacteriocin ISK–1 is very similar to that of lacticin 481 from *Lactococcus lactis*. This result suggests that bacteriocin ISK–1 could be a novel lacticin–481 type lantibiotic.

DISCUSSION

The purification of bacteriocin ISK–1 described here resulted in 30–fold increase in the specific activity with recovery of 17% of the activity. Bacteriocin ISK–1 has a tendency to aggregate with other proteins. Hence, after ammonium sulfate precipitation, the precipitate with antimicrobial activity could not be dissolved during dialysis against 0.01 N HCl and any other physiological buffer solutions. In order to solubilize the aggregates, 6 M urea–HCl (pH 3.0) was used, and no inactivation of bacteriocin ISK–1 was observed. Consequently, the total bacteriocin activity was higher than that assayed in the initial supernatant. This phenomenon was described in other bacteriocins (Joeger and

Klaenhammer, 1986; Mørtvedt *et al.*, 1991; Piard *et al.*, 1992; Jimenez–Diaz *et al.*, 1995). This could be attributed to the dissociation of the bacteriocin aggregates into the more active forms, for example, the monomer form.

In this study, we have a strong evidence for the presence of lanthionine and/or 3–methyllanthionine in bacteriocin ISK–1 by amino acid composition analysis. It is suggested that bacteriocin ISK–1 is a kind of lantibiotic. Furthermore, the first seven N–terminal amino acid sequence revealed significant homology to that of lacticin 481 (van den Hooven *et al.*, 1996) and variacin (Pridmore *et al.*, 1996). Judging from the results described and the antimicrobial spectrum (Kimura *et al.*, 1997b), it could be concluded that bacteriocin ISK–1 is a novel lacticin–481 type lantibiotic. Furthermore, we are presently attempting to perform the cloning and the sequencing of the corresponding structural gene in order to confirm the primary structure of bacteriocin ISK–1.

ACKNOWLEDGEMENTS

We thank Dr. Hayashi, K. Ogawa, and Ms. A. Kohno, Asahi Chemical Industries, Co., Ltd., Tokyo., Japan, for their kind support in the structure analysis of bacteriocin ISK–1.

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