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Kimura, Hirokazu
Laboratory of Microbial Technology, Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Nagano, Rie
Laboratory of Microbial Technology, Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Matsusaki, Hiromi
Laboratory of Microbial Technology, Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Sonomoto, Kenji
Laboratory of Microbial Technology, Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

他

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A Strain *Pediococcus* sp. ISK-1 Isolated from *Nukadoko* Produces a Novel Bacteriocin

Hirokazu Kimura, Rie Nagano, Hiromi Matsusaki, Kenji Sonomoto and Ayaaki Ishizaki

Laboratory of Microbial Technology, Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-81, Japan

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Pediococcus sp. ISK-1 isolated in our laboratory from well-aged *Nukadoko*, produces a bacteriocin which has a unique antimicrobial spectrum among pediocins. The antimicrobial activity was detected at the late exponential growth phase during fermentation and reached the maximum at the late stationary phase. The bacteriocin was stable at acidic pH, and more than 60% of the antimicrobial activity still remained even after being autoclaved at 121 °C for 20 min in the pH range of 3 to 8. This is the first report dealt with bacteriocin produced by lactic acid bacteria isolated from *Nukadoko*.

INTRODUCTION

Many lactic acid bacteria are industrially important microorganisms, and they are used in traditional fermented foods. Some lactic acid bacteria produce several antimicrobial substances including organic acids, hydrogen peroxide, and bacteriocins which are responsible for antagonistic activity. It is reasonable that these antimicrobial substances may contribute to the storage of fermented foods and the stabilization of the microflora. The preservative effect of lactic acid bacteria has been investigated to enhance the quality of foods. Bacteriocins from lactic acid bacteria are proteinaceous compounds which generally exhibit bactericidal mode of action against Gram-positive bacteria that are closely related to bacteriocin-producing species (Tagg *et al.*, 1976). Among bacteriocins produced by lactic acid bacteria, nisin and pediocin have been genetically and biochemically well-investigated (Klaenhammer, 1993). Nisin has been approved by the World Health Organization as a preservative in the food industry. Recently, we have succeeded in purification of bacteriocin from the culture broth of *Lactococcus lactis* IO-1 (JCM7638) isolated in our laboratory (Matsusaki *et al.*, 1996a). The bacteriocin was identical with nisin Z, a natural nisin variant. Furthermore, we have successfully achieved nisin Z fermentative production, resulting in about three-fold yield of the peptide (Matsusaki *et al.*, 1996b).

Pediocin is produced by *Pediococcus* sp. which are widely associated with the fermentation of meat and vegetables. The two principal species, *P. acidilactici* and *P. pentosaceus*, have been found to produce bacteriocins, e. g. pediocin AcH (Bhunia *et al.*, 1988), pediocin PA-1 (Henderson *et al.*, 1992), pediocin SJ-1 (Schved *et al.*, 1993), and pediocin L50 (Cintas *et al.*, 1995) which were purified from fresh and fermented sausage. These bacteriocins have molecular mass of 2,700 to 4,600 Da and are sensitive to proteolytic enzymes. Pediocin is resistant to heat and some organic solvent and is active

over a wide range of pH. The antimicrobial activity of pediocin occurs through changes in the membrane potentials of indicator microorganisms, which leads to a collapse of proton motive force, an efflux of intracellular ions and cell death (Bhunia *et al.*, 1991; Christensen and Hutkins, 1992; Chikindas *et al.*, 1993; Chen and Montville, 1995). The wide antimicrobial spectrum against food-borne pathogens and spoilage microorganisms such as listeria and clostridia enables pediocin to be used as a natural food biopreservative (Pucci *et al.*, 1988; Nielsen *et al.*, 1990; Foegeding *et al.*, 1992).

A quite unique new strain, *Pediococcus* sp. ISK-1 was isolated in our laboratory from well-aged *Nukadoko*, the bed of fermented rice bran where exist not only yeast but a variety of Gram-positive bacteria. Salt-tolerant bacteria such as *Lactobacillus* and *Pediococcus* are known to be associated with *Nukadoko*. A homofermentative lactic acid bacterium, strain ISK-1 utilize xylose as well as glucose as carbon source but not lactate. Moreover, this strain was proved to grow well under 18% NaCl (Ishizaki *et al.*, unpublished results). It is reasonable to assume that some of the lactic acid bacteria associated with *Nukadoko* might be able to produce bacteriocins which maintain the quality of *Nukadoko* for a long time. However, few results are available in the literature for bacteriocins produced by salt-tolerant lactic acid bacteria from *Nukadoko*. This paper deals with partial characterization of the bacteriocin from strain ISK-1. The results showed that this bacteriocin, designated ISK-1 bacteriocin, was probably a novel pediocin compared to the others reported to date.

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. *Bacillus subtilis* C1 (Ishizaki *et al.*, 1992a), which was also isolated in our laboratory, was used as indicator microorganism for assays of antimicrobial activity. *B. subtilis* C1 was aerobically cultivated at 30°C for 18 h in bouillon liquid medium containing 0.7% meat extract, 1.0% polypeptone and 0.5% NaCl. For the production of ISK-1 bacteriocin, the stock culture of *Pediococcus* sp. ISK-1 was statically subcultured in 10 ml of thioglycolate medium without glucose (TGC medium; Difco Laboratories, USA) at 37°C for 18 h and then transferred to 100 ml of the following medium, referred to as CMG medium. CMG medium contained 1.0% glucose, 0.5% yeast extract, 0.5% polypeptone and 0.5% NaCl in distilled water at pH 7.0 for preculture. An aliquot of 25 ml of the preculture, after growth at 37°C for 6 h, was inoculated into a 1-liter jar fermentor with a working volume of 500 ml of CMG 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 concentrations was analyzed according to the method described previously (Ishizaki *et al.*, 1992b), and lactate concentration was determined by the high performance liquid chromatography (638-30 HPLC., Hitachi Co. Ltd., Japan) with a spectrophotometric detector (SPD-10AV, Shimazu Co. Ltd., Japan) at 210 nm. The

analytical conditions were as follows: column, Aminex HPX-87H (0.78×30 cm, Bio-Rad Co. Ltd., USA); column temperature, 65 °C; solvent for elution, 50 mM H_2SO_4 solution; flow rate, 0.2 ml/min (Masson *et al.*, 1991).

Bacteriocin preparation

The culture supernatant of *Pediococcus* sp. ISK-1 from a jar fermentor 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 90% saturation by the addition of solid ammonium sulphate and allowed to stand overnight at 4 °C. The precipitate was collected by centrifugation at 16,000×g for 20 min and then suspended in a small volume of 0.01 N HCl. This suspension was dialyzed against 20 mM Tris-HCl buffer (pH 7.6) in a Spectra/Por membrane (Spectrum Medical Industries, Inc., USA; molecular weight cut off, 1,000). Twenty ml of the active dialyzate was obtained. Unless otherwise noted,the concentrated and desalted ISK-1 bacteriocin sample was used to investigate the characterization.

Bioassay

The antimicrobial activity was detected by the two bioassay methods using turbidimetric assay and agar disc diffusion assay. The turbidimetric assay was performed in test tubes containing 5 ml of the assay medium (10-fold diluted CMG medium) buffered with 20 mM sodium phosphate buffer (pH 7.0). Fifty microliter of 100-fold diluted culture of *B. subtilis* C1 grown for 18 h at 30 °C with shaking (120 strokes/min) was inoculated into each test tube, and then an appropriate volume of the ISK-1 bacteriocin sample was added aseptically. The test tubes containing assay mixture were incubated at 30 °C for 18 h. The absorbance at 562 nm of the assay medium was measured to confirm the growth inhibition of the indicator strain *B. subtilis* C1.

Agar disc diffusion 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 (Oxoid, England) or bouillon soft agar media (0.7% agar). The mixture was poured into petridishes. After solidification of the soft agar media, sterile paper discs (10 mm diameter) with the original or a serial 2-fold diluted sample of ISK-1 bacteriocin were placed on the surface. After the plates were incubated for 18 h at the optimum temperature for each indicator microorganism, inhibitory zones formed around the paper discs were observed.

For the turbidimetric assay, the antimicrobial activity was defined as a nisin converting unit (NCU) in which 1 NCU is equivalent to the activity of 1 g of commercial nisin (ICN Biomedicals Inc., USA; activity, 1,000 U/mg-solid).

pH and heat stability of ISK-1 bacteriocin

ISK-1 bacteriocin sample was exposed to various pHs at 4 °C or 30 °C for 24 h. The bacteriocin was also autoculaved at 121 °C for 20 min. The each sample was adjusted to pH 3.0 with HCl or NaOH after the treatments, and then the antimicrobial activity was determined by a turbidimetric assay.

Effects of enzyme treatment on antimicrobial activity

The enzymes, acid protease (Sigma, USA), trypsin (Sigma), α-chymotrypsin (Sigma), proteinase K (Merck, Germany), pepsin (Nacalai Tesque, Japan), ficin (Sigma), papain (Merck), Actinase E (Kaken Seiyaku, Japan), pancreatin (Nacalai Tesque), lysozyme (Seikagaku Kogyo, Japan), lipase (Sigma), ribonuclease A (Sigma) and α-amylase (Katayama Kagaku, Japan) were dissolved at a concentration of 2 mg/ml in the following buffer; acid protease and pepsin in 70 mM Sørensen buffer (disodium hydrogen citrate-HCl) at pH 2.0 and pH 2.8, respectively, papain and lysozyme in 70 mM phosphate buffer at pH 6.0, trypsin, α-chymotrypsin, proteinase K, actinase E, ficin, pancreatin, lipase, ribonuclease A and α-amylase in 70 mM phosphate buffer at pH 7.0. Two milliliters of ISK-1 bacteriocin sample were loaded on a tC_{18} Sep-Pak cartridge (Waters, USA), 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. The eluate was lyophilized and then the active material was dissolved in 1 ml of each buffer. The bacteriocin sample was added to 1 ml of each enzyme reaction solution, and the reaction mixture was incubated for 24 h at the indicated temperature of each enzyme. The each antimicrobial activity was then determined by a turbidimetric assay. Reaction mixtures without enzymes were used as positive controls.

Antimicrobial spectrum of ISK-1 bacteriocin

An agar disc diffusion assay was used to investigate the antimicrobial spectrum of the bacteriocin produced by strain ISK-1. The microorganisms tested were *P. acidilactici* JCM 5885^T, *B. subtilis* C1 (Ishizaki *et al.*, 1992a), *P. pentosaceus* JCM 5809^T, *Lactobacillus plantarum* JCM 1057^T, *Lactobacillus casei* ssp. *casei* JCM 1134^T, *Lactococcus lactis* IO-1 (nisin Z producing-strain isolated in our laboratory (Matsusaki *et al.*, 1995)), *Lactococcus lactis* JCM 5805^T, *Lactococcus lactis* ssp. *cremoris* TUA 13442 and *M. luteus* IFO 12708. MRS, LB and bouillon agar plate media were used for lactic acid bacteria, *M. luteus* and *B. subtilis*, respectively. The each plate was incubated for 18 h at the optimum temperature of the microorganism tested.

RESULTS

ISK-1 bacteriocin production

As a result of preliminary work, a peptide antibiotic was partially purified from *Nukadoko* by ammonium sulphate precipitation, two times gel filtration chromatography with a Sephadex G-50 and reverse-phase chromatography (data not shown).

Relationship between the growth and the bacteriocin production of strain ISK-1 was investigated in CMG medium. The antimicrobial activity of the culture supernatant was determined by a turbidimetric assay after it was desalted by Micro Acylizer G1 (Asahikasei Co. Ltd., Japan) to remove the inhibitory effect of organic acids. As shown in Fig. 1, the antimicrobial activity of the culture supernatant was detected at the late exponential phase of growth, and a maximum level of 0.232 NCU/*l* was obtained at the end of the stationary phase of growth.

pH and heat stability of ISK-1 bacteriocin

pH and heat stability of ISK-1 bacteriocin was examined by a turbidimetric assay. As

shown in Fig. 2, in spite of instability at 30 °C, the storage at 4 °C for 24 h resulted in a good stability in the pH range of 2 to 12. At 121 °C, more than 60% of the antimicrobial activity still retained in the pH range of 3 to 8. This indicates that the bacteriocin is heat stable.

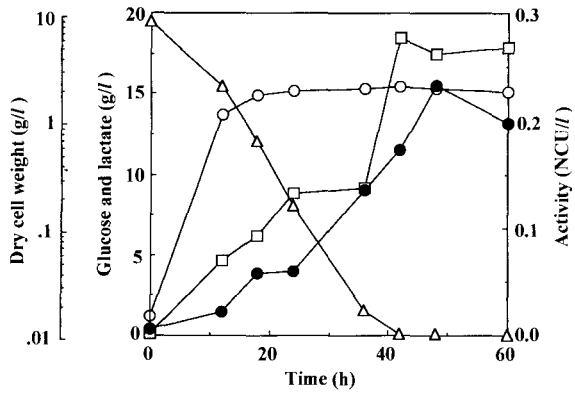

Fig. 1. Batch Fermentation Profile of *Pediococcus* sp. ISK-1.
The pH-controlled fermentation was carried out as described in Materials and Methods.

cell growth (○), residual glucose concentration (△), lactate production (□) and activity of bacteriocin (●).

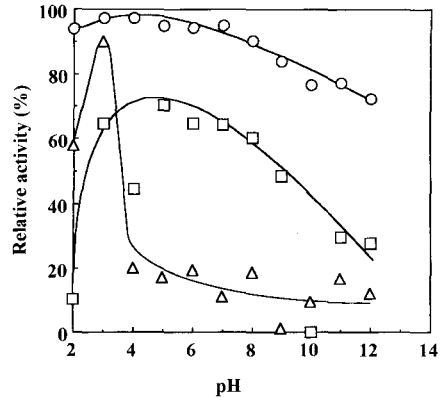

Fig. 2. pH and Heat Stability of the Bacteriocin Produced by *Pediococcus* sp. ISK-1.

Symbols: stored at 4 °C for 24 h (○); stored at 30 °C for 24 h (△); autoclaved at 121 °C for 20 min (□). The fresh ISK-1 bacteriocin sample having the activity of 0.199 NCU/*l* was defined as 100%.

Effects of enzyme treatment on the antimicrobial activity

The effects of enzyme treatment on the antimicrobial activity of ISK-1 bacteriocin were examined by a turbidimetric assay. The bacteriocin was inactivated by acid protease, α-chymotrypsin, pepsin, ficin and papain, while it was not affected by lysozyme, lipase, ribonuclease A and α-amylase (Table 1). This suggests that the antimicrobial substance produced by *Pediococcus* sp. ISK-1 could be a proteinaceous inhibitory substance, that is a bacteriocin (Tagg *et al.*, 1976).

Table 1. Effects of Enzyme Treatment on the Antimicrobial Activity of the Bacteriocin Produced by *Pediococcus* sp. ISK-1

Enzyme	Maker	Activity[a]	Reaction temperature (°C)
Acid protease	Sigma, USA	−	37
Trypsin	Sigma	+	25
α-Chymotrypsin	Sigma	−	25
Proteinase K	Merck, Germany	+	37
Pepsin	Nakalai Tesque, Japan	−	37
Ficin	Sigma	−	37
Papain	Merck	−	40
Actinase E	Kaken Seiyaku, Japan	−	40
Pancreatin	Nakalai Tesque	−	37
Lysozyme	Seikagaku Kogyo, Japan	+	35
Lipase	Sigma	+	37
Ribonuclease A	Sigma	+	37
α-Amylase	Katayama Kagaku, Japan	+	37

ISK-1 bacteriocin was partially purified as described in Materials and Methods and then used in this experiment.

[a]Antimicrobial activity was determined by a turbidimetric assay. (+, presence and −, loss of activity)

Antimicrobial spectrum of ISK-1 bacteriocin

A more sensitive indicator microorganism to ISK-1 bacteriocin should be used for the purification and the investigation of the characterization. *P. acidilactici* JCM 5885^T was the most sensitive to ISK-1 bacteriocin among the microorganisms tested in this study (Table 2). It was found that ISK-1 bacteriocin inhibited the growths of neither *Lactobacillus plantarum* nor *P. pentosaceus*, although established pediocins inhibited growth of them (Bhunia *et al.*, 1988; Henderson *et al.*, 1992; Schved *et al.*, 1993; Cintas *et al.*, 1995). The clear inhibitory zone of ISK-1 bacteriocin against *P. acidilactici* JCM 5885^T and *M. luteus* IFO 12708 are shown in Fig. 3.

Table 2. Antimicrobial Spectrum of the Bacteriocin from *Pediococcus* sp. ISK-1

Indicator organisms	Inhibition
Pediococcus acidilactici JCM 5885[T]	+++
Pediococcus pentosaceus JCM 5809[T]	−
Lactobacillus plantarum JCM 1057[T]	−
Lactobacillus casei ssp. *casei* JCM 1134[T]	++
Lactococcus lactis IO-1[a]	−
Lactococcus lactis JCM 5805[T]	++
Lactococcus lactis ssp. *cremoris* TUA 13442	−
Micrococcus luteus IFO 12708	++
Bacillus subtilis C1	+

ISK-1 bacteriocin sample used here was dialyzed as described in the text.
[a] Nisin Z-producing strain isolated in our laboratory (Matsusaki *et al.*, 1996a).
+++: Large inhibitory zone (>20 mm)
++: Medium inhibitory zone (15-20 mm)
+: Small inhibitory zone (10-15 mm)
−: No inhibitory zone
JCM: Japan Collection of Microorganisms.
IFO: Institute for Fermentation, Osaka, Japan.
TUA: Tokyo University of Agriculture, Japan.

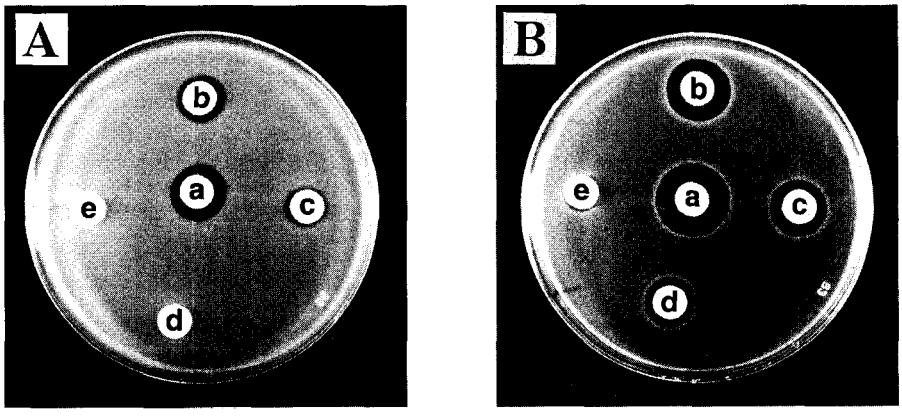

Fig. 3. Inhibitory Zones of Bacteriocin of *Pediococcus* sp. ISK-1 against *Micrococcus luteus* IFO 12708 (A) and *Pediococcus acidilactici* JCM 5885[T] (B).

Discs contained 40 μl of each sample. (a), original; (b), 2-fold diluted; (c), 4-fold diluted; (d), 8-fold diluted; (e), 16-fold diluted

DISCUSSION

Nukadoko, well-aged and kept over several hundred year is attractive as a source for unique lactic acid bacteria differing in characteristics such as carbohydrate utilization and bacteriocin production. The formation of some kinds of bacteriocin as well as acidic end-

products, primarily lactic acid, from the fermentation of carbohydrate-rich substrates such as rice bran is largely responsible for creating an environment unfavorable for the growth of spoilage organisms. Little work has been done to find out bacteriocin-producing lactic acid bacteria from *Nukadoko*. In this paper, we clarified the isolate, *Pediococcus* sp. ISK-1 to produce a bacteriocin and also characterized it.

The bacteriocin of strain ISK-1 which was dialyzed after ammonium sulphate precipitation as described in Materials and Methods, was unstable at 30°C (Fig. 2) since a lot of extracellular proteases might be secreted and present in the bacteriocin sample. This should be predictable from the result that the partially purified bacteriocin was stable at 35-37°C for 24 h without many kinds of protease (Table 1). Furthermore, autoclave treatment of the dialyzed bacteriocin resulted in not only a good stability of the peptide but a denaturation of the protease contaminants. Heat stability of ISK-1 bacteriocin over a wide range of pH is the characteristic of pediocin produced by some strains of *Pediococcus* sp (Klaenhammer, 1993). Nisin is used as a food preservative in many countries (Delves-Broughton, 1990). However, the application in foods is limited because of the pH stability. Therefore, ISK-1 bacteriocin may be preferable for the commercial application.

Moreover, the antimicrobial spectrum of ISK-1 bacteriocin suggests that it might be different from pediocins published to date. To ensure whether it is a novel pediocin or not, it is necessary to purify the bacteriocin of strain ISK-1 and to characterize it in detail.

The genetic determinants for both pediocin production and immunity are linked to plasmid DNA (Klaenhammer, 1993). It was found that strain ISK-1 possessed at least two plasmids (53 kbp and 2 kbp) and these plasmids were designated pPI1 and pPI2, respectively (data not shown). No deletion of antimicrobial activity occurred through curing plasmid pPI2 by growth at elevated temperature. Thus plasmid pPI2 was not linked to the bacteriocin production. It has not been proved whether plasmid pPI1 is linked to the bacteriocin production or not. At least, the plasmid sizes of strain ISK-1 were quite different from those of pediocin-producing strains (Bhunia *et al.*, 1988; Henderson *et al.*, 1992; Schved *et al.*, 1993; Cintas *et al.*, 1995).

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