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Purification and Characterization of a Novel Class IIa Bacteriocin, Piscicocin CS526, from Surimi-Associated *Carnobacterium piscicola* CS526

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The bacteriocin piscicocin CS526 was inactivated by proteolytic enzymes, was stable at 100°C for 30 min, had a pH range of 2 to 8, and was active against *Enterococcus*, *Listeria*, *Pediococcus*, and *Leuconostoc*. The N-terminal sequence was YGNGL, not the YGNGV consensus motif common in class IIa bacteriocins (alternate residues underlined). The molecular mass of piscicocin CS526, which had a bactericidal mode of action, was ~4,430 Da.

Bacteriocins are ribosomally synthesized proteins that inhibit bacteria closely related to the producer strain, food-borne pathogens, and spoilage bacteria (12, 18). *Carnobacterium piscicola* CS526 inhibits *Listeria monocytogenes* growth in cold-smoked salmon (21). Several bacteriocin-producing carnobacteria have been reported (1, 4, 10, 11, 13, 17). The objectives of this study were to purify and characterize piscicocin CS526.

Preliminary characterization of the bacteriocin. To determine the effects of enzymes on the bacteriocin, the cell supernatants (180 μl) of the culture neutralized with NaOH were incubated with 20 μl of enzyme solutions (10 mg/ml in 50 mM phosphate buffer [pH 7.0]) at 35°C for 1 h. After boiling for 5 min, the residual activity was determined by the agar well diffusion assay (21). The inhibitory activity was completely destroyed by α-chymotrypsin, papain, proteinase K, and actinase and partly inhibited by trypsin (67% of residual activity). Other enzymes, such as catalase, RNase, and lipase, did not affect the activity.

To determine the thermal stability, the cell supernatant of the neutralized culture was heated for 15 or 30 min at 70, 80, 90, 100, and 121°C and then assayed for activity. The inhibitory activity was completely stable after 30 min at 100°C but was partially inactivated at 121°C for 15 min (44% of residual activity).

To determine the effect of pH on bacteriocin activity, 50 μl of the cell supernatant was added to 950 μl of tryptic soy broth with 0.6% yeast extract (TSBYE) adjusted with NaOH or HCl to different pH values. The samples were assayed for activity after 1 h at 25°C. The activity was completely stable at pH 2 to 8 but reduced at pH values of 9 to 11 (67, 44, and 30% of residual activity at pH 9, 10, and 11, respectively).

The antagonistic effect of cell supernatant of an overnight culture of CS526 in TSBYE against 27 strains of gram-positive bacteria and 6 strains of gram-negative bacteria was determined by the agar well diffusion assay. *Enterococcus faecalis* JCM8726, *Enterococcus faecium* JCM8727, *Enterococcus durans* JCM8725, *Enterococcus hirae* JCM8729, *Pediococcus pentosaceus* JCM5890, *Tetragenococcus halophilus* JCM5888, and *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM6124 were inhibited. *L. monocytogenes* IID578 and IID581, *Listeria innocua* FTHU8221, and *Listeria grayi* subsp. *murrayi* FTHU204 were also inhibited. Gram-positive spore-forming bacteria (*Bacillus cereus* JCM2152, *Bacillus subtilis* IFO13719, *Bacillus megaterium* IAM13418, *Bacillus licheniformis* IFO12107, *Bacillus sphaericus* IAM13420, and *Paenibacillus polymyxa* JCM2507) and gram-negative bacteria (*Escherichia coli* IFO15034, *Salmonella enterica* serovar Enteritidis RIMD1933001, *Pseudomonas fluorescens* JCM5963m and *Aeromonas hydrophila* IFO3820) were not inhibited. These results demonstrated that the inhibitory activity of the *C. piscicola* CS526 was due to a proteinaceous molecule exhibiting excellent heat stability. Therefore, the antilisterial substance produced by *C. piscicola* CS526 was confirmed as a bacteriocin and the bacteriocin has been named piscicocin CS526.

Mode of action. The effect of the bacteriocin on nongrowing cells of *L. monocytogenes* IID581 and *E. hirae* JCM8729 was studied in TSBYE. Both strains were grown at 30°C for 16 h in TSBYE, harvested by centrifugation, and resuspended in fresh TSBYE (pH 7.0) to ~10^6 cells/ml. The filter-sterilized cell supernatant of the *C. piscicola* CS526 culture was immediately added to final concentrations of 600 arbitrary units (AU)/ml, and the cells were incubated at 30°C. The viable cell counts of *L. monocytogenes* and *E. hirae* decreased quickly from 8.0×10^5 and 1.7×10^6 CFU/ml to 1.5×10^4 and 1.4×10^4 CFU/ml, respectively, after the treatment (30 min) with piscicocin CS526 (Fig. 1), indicating that piscicocin CS526 is bactericidal rather than bacteriostatic.

Bacteriocin purification and molecular properties. Bacteriocin was purified from the supernatant of a 2-liter *C. piscicola* CS526 culture propagated at 30°C for 12 h. Bacteriocin was precipitated with ammonium sulfate (50%[wt/vol]), collected by centrifugation (15,000 × *g*, 30 min, 4°C), and solubilized in 20 mM sodium phosphate buffer (pH 6.0) (buffer A). The sample was applied onto a 300-ml Sephadex G50 (Amersham Biosciences, Tokyo, Japan) gel-filtration gel equilibrated with buffer A, and the bacteriocin was eluted. The active fraction

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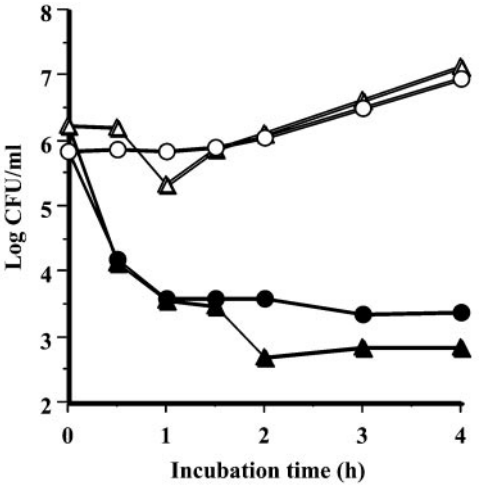

FIG. 1. Survivor curve of *L. monocytogenes* IID581 (circles) and *E. hirae* JCM8729 (triangles) in the presence of piscicocin CS526. The concentrations of the bacteriocin were 0 (open symbols) and 600 (closed symbols) AU/ml.

was applied onto a 30-ml SP-Sepharose Fast Flow (Amersham Biosciences) cation-exchange column equilibrated with 25 mM sodium *N*-morpholinoethanesulfonic acid (MES) containing 3.0 M urea, pH 6.0. (buffer B). After the column was washed with 120 ml of buffer B, the bacteriocin was eluted with linear NaCl gradient (0 to 0.5 M) at pH 6.0, and loaded on a Sep-Pack C_{18} cartridge (Waters, Milford, Mass.). The cartridge was washed with 20% 2-propanol in 0.1% trifluoroacetic acid (TFA), and the bacteriocin was eluted with 80% 2-propanol in 0.1% TFA. After drying, the bacteriocin was dissolved in 0.05% TFA and applied to C_{18} reverse-phase column (ODS-80Ts; 4.6 by 150 mm; Tosho, Tokyo, Japan) connected to a high-performance liquid chromatograph. Elution was performed by using a linear gradient from 100% 0.05 TFA to 100% 2-propanol in 60 min at a flow rate of 0.5 ml/min.

The purification was approximately a 1.6×10^4-fold overall, with a 7% yield. The inhibitory fraction after reverse-phase high-performance liquid chromatography (RP-HPLC) was analyzed further by Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methods (5). SDS-PAGE revealed a single band of protein (Fig. 2D, lane 2) that inhibited *L. monocytogenes* IID581 (Fig. 2D, lane 3). It was estimated to be a 4.0- to 4.5-kDa peptide.

The purified piscicocin CS526 was subjected to mass spectral

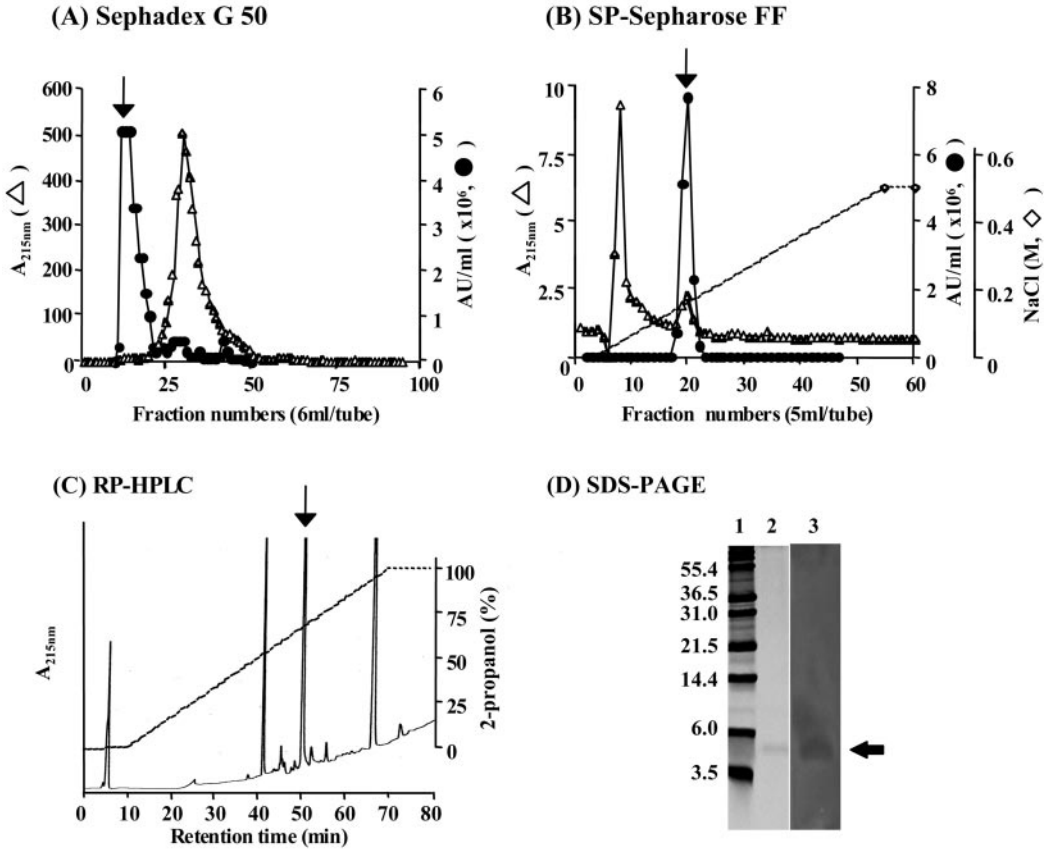

FIG. 2. Purification of piscicocin CS526 by gel filtration (A), cation-exchange (B) and RP-HPLC (C), and antibacterial detection SDS-PAGE of piscicocin CS526 (D). Purified piscicocin CS526 was analyzed by SDS-PAGE. The gel was removed and cut into two parts. The first half, containing molecular weight markers (lane 1) and the purified piscicocin CS526 (lane 2), was stained with silver. The other half, containing the purified bacteriocin (lane 3), was overlaid with *L. monocytogenes* IID581 and incubated at 30°C for 24 h. The arrows indicate the bacteriocin activities.

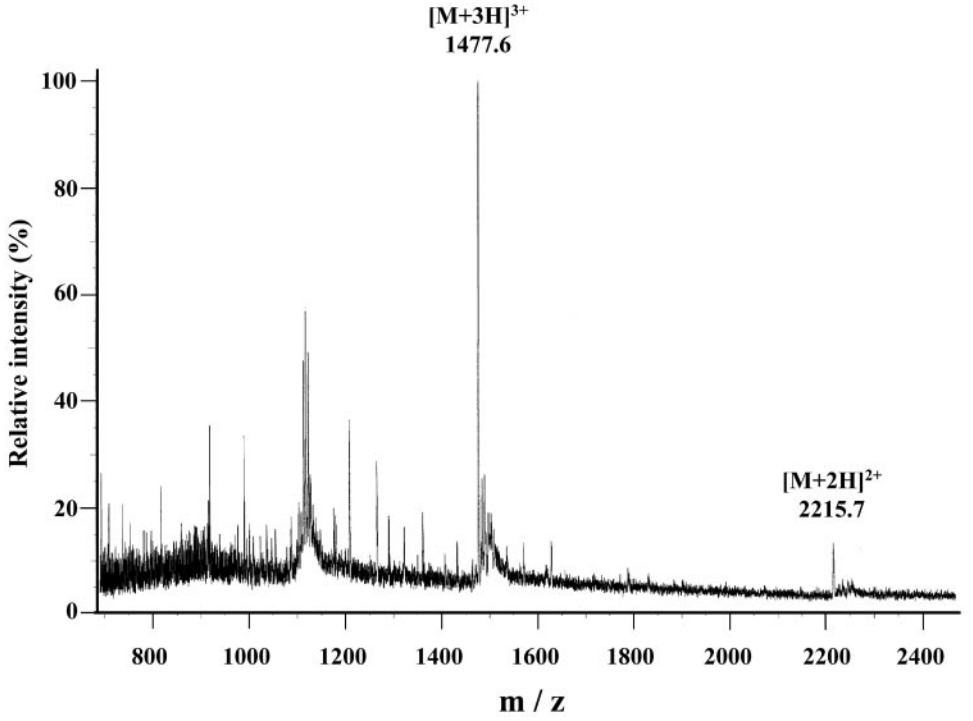

FIG. 3. Electrospray ionization mass spectrometry analysis of purified piscicocin CS526.

measurement by electrospray ionization spectrometry (model JM-700TZ mass spectrometer); JEOL, Peabody, Mass. Two unequivocal pseudomolecular ions $[M + nH]^{n+}$ were observed at m/z corresponding to $n = 2$ and 3 protonated species, whose averaged molecular mass was 4,429.6 Da (Fig. 3).

The partial NH_2-terminal amino acid sequence, comprising 43 amino acid residues, was determined by Edman degradation with Procise 492-HT Protein Sequencer (Applied Biosystems, Foster City, Calif.) (Table 1). Piscicocin CS526 shared significant homology with munditicin and piscicolin 126, produced, respectively, by *Enterococcus mundtii* (3) and *C. piscicola* JG126 (11), belonging to class IIa bacteriocins. However, there were no identical sequences in the database. Class IIa bacteriocins, according to the system proposed by Klaenhammer (12), have antilisterial activity and possess the highly conserved N-terminal motif, YGNGV (15). Bhugaloo-Vial et al. (4) also indicated that it was possible to increase the consensus sequence as YGNGVXCX(K/N)XXCXV(N/D)(W/K/R)X(G/A/S)(A/N). Piscicocin CS526 exhibited strong antilisterial activity but possessed a novel N-terminal sequence that contained a change of Val to Leu at position 7. Only three other bacteriocins, bacteriocin 31 (19), sakacin 5X (20), and plantaricin C19 (2), possess this altered N-terminal sequence. The YGNGV consensus motif has been involved in a recognition step of the mechanism of action of class IIa bacteriocins (7, 8), but the YGNGV consensus motif did not appear to be involved in the initial binding step for pediocin PA-1 (6) Carnobacteriocin B2 activity was reduced by a substitution within the Y^3GNGV

TABLE 1. Sequence alignment of piscicocin CS526 produced by *C. piscicola* CS526 and other class IIa bacteriocins

Bacteriocin (reference)	Sequence and position[a]				
	1	10	20	30	40
Piscicocin CS526	KYYGNGLSXN	KKGXTVDWGT	AIGIIGNNAA	ANXATGGAAG	XNK
Mundticin (3)	KYYGNGVSCN	KKGCSVDWGK	AIGIIGNNSA	ANLATGGAAG	WSK
Piscicolin126 (11)	KYYGNGVSCN	KNGCTVDWSK	AIGIIGNNAA	ANLTTGGAAG	WNKG
Bavaricin A (12a)	KYYGNGVHXG	KHSXTVDWGT	AIGNIGNNAA	ANXATGXNAG	G
Pediocin PA-1 (9)	KYYGNGVTCG	KHSCSVDWGK	ATTCIINNGA	MAWATGGHQG	NHKC
Sakacin 5X (20)	KYYGNGLSCN	KSGCSVDWSK	AISIIGNNAV	ANLTTGGAAG	WKS
Bacteriocin 31 (19)	ATYYGNGLYCN	KQKCWVDWNK	ASREIGKIIV	NGWVQHGPWA	PR
Divercin V41 (13)	TKYYGNGVYCN	SKKCWVDWGQ	ASGCIGQTVV	GGWLGGAIPG	KC
Carnobacteriocin BMI (15)	AISYGNGVYCN	KEKCWVNKAE	NKQAITGIVI	GGWASSLAGM	GH
Carnobacteriocin B2 (16)	VNYGNGVSCS	KTKCSVNWGQ	AFQERYTAGI	NSFVSGVASG	AGSIGRRP
Piscicocin V1a (4)	KYYGNGVSCN	KNGCTVDWSK	AIGIIGNNAA	ANLTTGGAAG	WNKG
Bavaricin MN (11a)	TKYYGNGVYCN	SKKCWVDWGQ	AAGGIGQTVV	XGWLGGAIPG	K
Consensus	YGNG C	C			

[a] X indicates unidentified residues. The altered L residues and consensus motif residues are underlined.

motif (Tyr^3 to Phe) (16). Pediocin AcH activity was dramatically reduced by a mutation within the YGN^5GV motif (Asn^5 to Lys) (14). In this study, we found that piscicocin CS526, which possessed YGNGL sequence, had anti-*Listeria* activity typical of class IIa bacteriocins. Accordingly, the presence of Val^7 within the $YGNGV^7$ motif could not be a prerequisite for the antimicrobial activity of class IIa bacteriocins.

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