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Isolation and Characterization of Lacticin 10790, a New Bacteriocin Produced by *Lactococcus lactis* subsp. *cremoris* KFCC 10790

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Abstract A new bacteriocin, named lacticin 10790, was purified from *Lactococcus lactis* subsp. *cremoris* KFCC 10790 by sequential adsorption, immobilized metal-affinity, cation-exchange, and C_{18} reverse-phase chromatographies. The molecular mass of the bacteriocin was estimated to be between 3,000 and 3,500 Da. Lacticin 10790 showed a broad antimicrobial spectrum against many gram-positive bacteria. The bacteriocin was stable to heat and in the pH range between 2 and 6. Lacticin 10790 was destroyed by digestion with proteases and exhibited a bactericidal mode of action. An amino acid composition analysis of purified lacticin 10790 revealed a high concentration of hydrophobic amino acids. The N terminus of the bacteriocin was found to be blocked, upon analysis by Edman degradation. The results suggest that lacticin 10790 is a class I bacteriocin.

Key words: Bacteriocin, purification, *Lactococcus lactis* subsp. *cremoris*

Bacteriocins are proteinaceous compounds that exert a bactericidal action on closely related susceptible bacteria [11, 15]. Bacteriocins of lactic acid bacteria (LAB) have received considerable attention in recent years due to their present and potential applications in the food industry as natural preservatives [6, 7, 18] and in health and cosmetic products. Several bacteriocins from LAB have been previously identified and characterized. They appear to be heterogeneous in their molecular weights, biochemical properties, and activity spectra [10, 11, 15, 17]. On the basis of various biochemical and genetic studies, bacteriocins from LAB were classified by Klaenhammer [16] into four major groups: class I, lantibiotics, including lanthionine-containing bacteriocins such as nisin; class II, small heat-stable non-lantibiotics; class III, large heat-labile bacteriocins; and class IV, complex bacteriocins that require a lipid or carbohydrate moiety for their activity.

A number of *Lactococcus lactis* strains produce bacteriocins [11, 15, 17]. The best-characterized lactococcal bacteriocin is nisin, which is a 34-amino acid lantibiotic containing the unusual amino acids lanthionine and β-methyllanthionine [4, 13]. The inhibitory spectra of the different lactococcal bacteriocins are generally narrow. Thus, isolation and characterization of bacteriocins which have broad inhibitory spectra are important to develop new natural preservatives.

Lactococcus sp. KFCC 10790 isolated from raw milk [21, 22] was found to produce a new nisin-like bacteriocin, named lacticin 10790. This strain was identified to be *Lactococcus lactis* subsp. *cremoris* by a 16S ribosomal DNA sequence analysis [12]. In this report, we describe the isolation and characterization of lacticin 10790, which differed from nisin in inhibitory spectra and amino acid composition.

The bacteriocin activity was estimated using an agar well diffusion method [1]. *Lactobacillus plantarum* ATCC 8739 was used as an indicator strain for a routine bacteriocin assay. Activity was defined as the reciprocal of the highest two-fold dilution showing distinct growth inhibition of the indicator strain and was expressed as activity units (AU) per milliliter. The protein concentration was determined as described by Bradford [3] using bovine serum albumin as the standard. Specific activities were expressed as activity units per milligram of protein.

All the purification steps were performed at room temperature. The bacteriocins were purified from a 1-l culture of *L. lactis* KFCC 10790 grown in Tween-free MRS medium prepared from basal ingredients at 30°C until the early stationary phase. The bacteriocin activity was effectively recovered from the culture supernatants with the adsorber resin, Amberlite XAD-2 (Sigma, St Louis, U.S.A.). This is most likely due to the highly hydrophobic

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nature of lacticin 10790, which is a general characteristic of bacteriocins. Thus, samples were applied to a column (5.0 by 30 cm), washed with 0.1% trifluoroacetic acid (TFA), and elution was carried out with 80% ethanol in 0.1% TFA. The eluents were concentrated using a rotary evaporator at 50°C. Lacticin 10790 was separated by its ability to bind to an immobilized metal-affinity chromatography (IMAC) column [20]. Iminodiacetic acid (IDA) Sepharose 6B Fast Flow gel (Sigma) was saturated with 50 mM $CuSO_4$ solution and washed with 5 bed volume of water to remove any excess metal ions. After the sample application, the gel was washed with 50 mM sodium acetate buffer (pH 5.0) and the bacteriocin activity was eluted with 0.2 M imidazole buffer (pH 6.0) at a flow rate of 6.0 ml/min. The fractions containing inhibitory activity were dried under vacuum using a centrifugal concentration system (Centra-Vac™, Vision Scientific Co., Korea) and then dissolved in 50 mM sodium acetate buffer (pH 5.0). Upon treatment with IMAC, the specific activity increased by 29-fold. Although the recovered activity from IMAC was only 54%, the metal-affinity chromatography provided a convenient and profound increase in specific activity (from 185 to 3,460 AU/mg). Further purification was achieved by cation-exchange chromatography on CM-Sepharose (Pharmacia, Uppsala, Sweden). The bacteriocin concentrate was applied to a CM-Sepharose column (2.5 by 20 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) and the column was washed extensively. Elution was carried out using the linear gradient of 0–1.0 M NaCl. The bacteriocin activity was eluted with a broad single peak at about 0.5 M NaCl, and its specific activity from the CM-Sepharose was 347-fold higher than that in the initial culture supernatant. Thus, the specific activity significantly increased during this step of purification. Although most colored impurities were removed by CM-Sepharose chromatography, this step did not provide a high degree of purity. Therefore, the pooled active fractions from cation-exchange chromatography were loaded on C_{18} Sep-Pak cartridges (Waters Millipore Corp., Milford, U.S.A.) and washed with 30% methanol in 0.1% TFA. The bacteriocin activity was eluted with 50% methanol in 0.1% TFA. This extract was dried by evaporation in a vacuum centrifuge and resolubilized in 0.1% TFA. The final purification of lacticin 10790 was achieved by reverse-phase high-performance liquid chromatography (HPLC) using an analytical C_{18} column (μBondapak C_{18}, 3.9 × 300 mm; Waters). The reverse-phase chromatography was repeated twice, and resulted in a significant increase in specific activity. Gradient elutions with water-acetonitrile gradients (1 ml/min) containing 0.1% TFA were used. The elution consisted of isocratic 20% acetonitrile for 10 min, followed by a linear gradient to 50% acetonitrile over 30 min. The polypeptides detected by A_{220} were collected manually. Fractions with bacteriocin activity were concentrated by evaporation, and a final purification was achieved by a second cycle of C_{18} reverse-phase chromatography with a shallower acetonitrile gradient. The running conditions consisted of 30 to 40% acetonitrile over 20 min. The overall purification resulted in a 1,231-fold increase in the specific activity, with a recovery of about 5%. The results are summarized in Table 1.

An analysis of the purified lacticin 10790 preparation was carried out by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) [19]. After electrophoresis, the gels were either stained with Silver Stain (Bio-Rad Laboratories, Richmond, U.S.A.) or bioassayed by the method of Bhunia *et al.* [2]. The analysis of purified lacticin 10790 using silver-stained tricine-SDS-PAGE revealed a single band, indicating that the bacteriocin had been purified to homogeneity (Fig. 1A). The estimated molecular mass of the bacteriocin was between 3,000 and 3,500 Da. An identical gel overlaid with a soft MRS agar (0.7%) containing an indicator strain revealed a zone of inhibition corresponding to the appropriate lane (Fig. 1B).

Using a well diffusion assay, the antagonistic effect of lacticin 10790 against various gram-positive and gram-negative bacterial genera was tested. To quantitate the inhibitory activity, the diameter of the inhibition zone (in millimeters) was measured. The result is summarized in Table 2, which shows a reasonably wide spectrum of activity.

Table 1. Purification of lacticin 10790 from *L. lactis* KFCC 10790.

Purification step	Total activity (AU)[a]	Total protein (mg)	Specific activity (AU/mg)	Yield (%)	Purification (fold)
Culture supernatant	6.21×10^5	5131.10	1.21×10^2	100	1.0
Amberlite XAD-2	4.74×10^5	2560.30	1.85×10^2	76	1.5
IMAC[b]	3.38×10^5	97.70	3.46×10^3	54	29.0
CM-Sepharose	1.93×10^5	4.60	4.20×10^4	31	347.0
HPLC (C_{18} reverse-phase)[c]					
First run	6.70×10^4	0.65	1.03×10^5	11	851.0
Second run	3.12×10^4	0.21	1.49×10^5	5	1231.0

[a]AU, activity units.
[b]IMAC, immobilized metal-affinity chromatography
[c]HPLC, high performance liquid chromatography

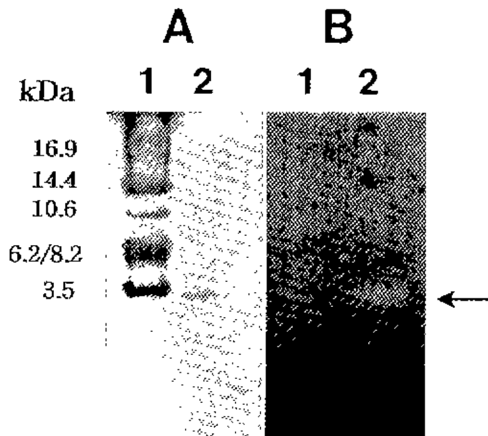

Fig. 1. Tricine-SDS-PAGE of lacticin 10790 and detection of antimicrobial activity.
(A) Silver-stained gel Lane 1, size standards (sizes indicated on the left) Lane 2, purified lacticin 10790 sample. (B) Overlaid gel with MRS soft agar containing *L. plantarum*. The clear zone (indicated by an arrow) represents the antimicrobial activity of lacticin 10790.

The activity spectrum includes many species in the genera of *Bacillus*, *Lactobacillus*, *Corynebacterium*, *Enterococcus* and *Listeria*, however, the gram-negative bacteria (except *Pseudomonas fluorescens*) were not affected by lacticin 10790. Usually, bacteriocins only inhibit closely related bacteria. One exception is nisin, which inhibits strains of various gram-positive genera, including the spore formers *Bacillus* and *Clostridium*.

The thermostability was assayed by an analysis of the residual activity in a lacticin 10790 sample after incubation at pHs 2 and 5 in a boiling water bath for up to 120 min and autoclaving at 121°C for 10 min. Lacticin 10790 was highly thermostable; It retained its antimicrobial activity even after heating at 100°C for 120 min or at 121°C for 10 min (Table 3). At 100°C, the half-life was found to be about 30 min. Therefore, lacticin 10790 belongs to the heat-stable group of bacteriocins. Temperature stability is very convenient if the bacteriocin is to be used as a food preservative, because many processing procedures involve

Table 2. Inhibitory spectrum of purified lacticin 10790 for various bacteria.

Indicator strain[a]	Cultivation medium[b]	Incubation temp. (°C)	Zone of inhibition (mm)[c]
Gram-positive bacteria			
Bacillus cereus KCTC 1012	NA	30	NIZD
Bacillus macerans IMSNU 10028	NA	37	7.0
Bacillus megaterium KCTC 3007	NA	30	8.0
Bacillus polymyxa IMSNU 12071	NA	30	NIZD
Bacillus pumilus KCTC 3348	NA	30	9.5
Bacillus subtilis KCTC 1028	NA	30	NIZD
Corynebacterium xerosis KCTC 3435	NA	30	10.5
Enterococcus faecium ATCC 11575	BHI	37	10.0
Lactobacillus brevis KCTC 3102	MRS	37	15.5
Lactobacillus casei KCTC 3109	MRS	30	12.0
Lactobacillus gasseri ATCC 33323	MRS	30	10.0
Lactobacillus plantarum ATCC 8739	MRS	30	16.5
Lactococcus lactis ATCC 7962	MRS	30	NIZD
Listeria monocytogenes KCTC 3444	BHI	37	8.5
Propionibacterium acnes ATCC 6919	BHI	37	7.0
Staphylococcus aureus ATCC 6538	NA	27	NIZD
Staphylococcus epidermis KCTC 1917	NA	37	NIZD
Streptococcus mutans KCTC 3283	BHI	37	NIZD
Gram-negative bacteria			
Enterobacter aerogenes KCTC 2190	NA	30	NIZD
Escherichia coli ATCC 8739	NA	37	NIZD
Klebsiella pneumoniae KCTC 2208	NA	37	NIZD
Proteus vulgaris KCTC 2512	NA	37	NIZD
Pseudomonas fluorescens IMSNU 10205	NA	37	12.5
Pseudomonas aeruginosa ATCC 9027	NA	37	NIZD
Salmonella typhimurium KCTC 1925	NA	37	NIZD
Serratia marcescens IMSNU 10257	NA	37	NIZD
Shigella sonnei KCTC 2009	NA	37	NIZD

[a]KCTC, Korean Collection for Type Cultures: IMSNU, Culture Collection Center, Institute of Microbiology, Seoul National University; ATCC, American Type Culture Collection

[b]MRS medium and brain heart infusion (BHI) medium were obtained from Merck (Darmstadt, Germany); nutrient broth (NA) medium was obtained from Sigma (St Louis, U.S.A.).

[c]Diameter of inhibition zone detected using 50 µl of purified lacticin 10790 (640 AU/ml) by the agar well diffusion method. NIZD, no inhibition zone detected.

Table 3. Heat stability of lacticin 10790 activity.

Treatment	Activity (AU/ml)	
	pH 2.0	pH 5.0
Control	2,560	640
100°C		
10 min	2,560	640
30 min	1,280	320
60 min	640	320
120 min	640	160
121°C (autoclave)		
10 min	160	40

a heating step. Lacticin 10790 was also very stable under a series of different conditions including storage at room temperature, 4°C and -20°C, and treatment with organic solvents such as methanol, ethanol, and acetonitrile.

Purified lacticin 10790 was dissolved in 0.1 M buffers with different pH values ranging from 2 to 11 (glycine-HCl, pH 2 to 4; sodium acetate, pH 4 to 6; sodium phosphate, pH 6 to 8; glycine-NaOH, pH 8 to 11). After 24-h incubation at room temperature, the residual lacticin 10790 activity was assayed by the well diffusion test. Lacticin 10790 maintained its activity within a pH range of 2 through 11. Its activity was very stable at pH below 6. At an alkaline pH, there was a gradual decrease in activity and the activity was completely lost at pH 12 (data not shown). The mechanism of inactivation is unknown, however, it could be due to denaturation, chemical modification, or a combination of both.

To determine its sensitivity against various proteases and hydrolytic enzymes, the purified bacteriocin (160 AU/ml) was treated at 37°C for 4 h with proteinase K, subtilisin, pronase E, α-chymotrypsin, trypsin, and pepsin at a final concentration of 10 μg/ml in 10 mM Tris-HCl at pH 7.5. It was also treated at 37°C for 2 h with lysozyme, catalase,

Table 4. Effects of various enzymes on lacticin 10790 and nisin activities.

Enzyme	Residual activity (AU/ml)	
	Lacticin 10790	Nisin[a]
None	160	160
Proteinase K	0	0
Subtilisin	0	0
Pronase E	0	0
α-Chymotrypsin	0	40
Trypsin	160	160
Pepsin	160	160
Lysozyme	160	160
Catalase	160	160
DNase	160	160
RNase A	160	160
Lipase	160	160

[a]Commercial nisin was obtained from Aplin & Barrett Ltd. (Trowbridge, U.K.).

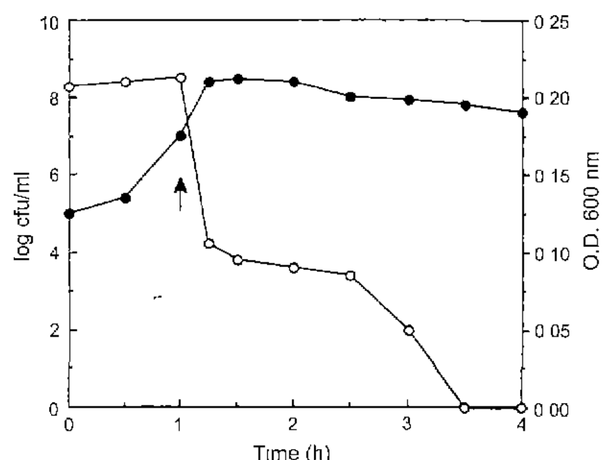

Fig. 2. Bactericidal effect of pure lacticin 10790 (0.1 μg/ml) on *L. plantarum*.

Viable cell counts (○) were determined by plating on an MRS agar. The optical density (●) was measured at 600 nm The arrow indicates the time point of bacteriocin addition

and lipase at a final concentration of 100 μg/ml, and with RNase A and DNase at a final concentration of 10 μg/ml in Tris-HCl, pH 7.5. The controls consisted of enzymes and bacteriocin, respectively, and the activity remaining in the sample after treatment was determined by agar well diffusion against a sensitive indicator strain. The results are shown in Table 4. The inhibitory activity of lacticin 10790 was completely eliminated after treatment with proteinase K, subtilisin, pronase E, and α-chymotrypsin, confirming a proteinaceous nature of the molecule. Trypsin and pepsin had no effect on the inhibitory activity. Other enzymes, including lysozyme, catalase, DNase I, RNase A, and lipase also had no effect on the activity of lacticin 10790.

To determine whether lacticin 10790 is bactericidal or bacteriostatic, lacticin 10790 (1,480 AU) was inoculated into an early log phase (A_{600}=0.17) culture of *L. plantarum*. After the addition of lacticin 10790, the mixture was incubated at 30°C. At appropriate time intervals, the mixture was examined by measuring its optical density at 600 nm. The number of surviving indicator bacteria was determined by plate counting (Fig. 2). Within 15 min after the addition of the bacteriocin, the viable colony counts dropped sharply to approximately 0.01%, and no viable cells were detected after 150 min. Killing, however, does not appear to cause cell lysis, as the absorbances of the treated suspensions were unchanged while the viabilities decreased by more than 99%. These data show that lacticin 10790 has a bactericidal mode of action, however, the mechanism of action is not yet known. Lantibiotics such as nisin have been shown to insert itself into the bacterial membrane, resulting in the formation of pores. This in turn leads to a loss of proton motive force and to subsequent lysis of the cell [9]. Other small, non-antibiotic bacteriocins such as pediocin are also known to form pores or channels in cell membranes [5].

The amino acid composition of pure lacticin 10790 indicates that it is different from nisin and other bacteriocins [10, 11, 15]. The amino acid analysis revealed a high molar concentration of hydrophobic amino acids, including Gly, Ala, Pro, Ile, and Leu, which accounted for the hydrophobic nature of lacticin 10790. In addition to normal amino acids, four unidentified compounds were observed in the acid hydrolysate (data not shown). These compounds may be the lanthionine or methyl-lanthionine. Attempts to determine its N-terminal sequence by Edman degradation were unsuccessful. This suggested that the amino-terminal might be blocked or, alternatively, that the peptide had a cyclic structure. Bacteriocins such as nisin [9], subtilin [8], and gallidermin [14] have been shown to contain unusual amino acids including lanthionine and dehydrobutyrine. Lanthionine results in blank cycles during sequencing by Edman degradation, whereas dehydrobutyrine produces a blockage of the reaction [14]. These data, together with its small size, hydrophobic nature, and thermostability, suggest that lacticin 10790 is a member of the class I bacteriocins, as defined by Klaenhammer [16].

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