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Enhanced Production, Purification, and Partial Characterization of Lacticin BH5, a Kimchi Bacteriocin Produced by *Lactococcus lactis* BH5

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Summary

Strain BH5 was isolated from naturally fermented Kimchi and identified as a bacteriocin producer, which has bactericidal activity against *Micrococcus flavus* ATCC 10240. Strain BH5 was identified tentatively as *Lactococcus lactis* by the API test and some characteristics. *Lactococcus lactis* BH5 showed a broad spectrum of activity against most of the non-pathogenic and pathogenic microorganisms tested by the modified deferred method. The activity of lacticin BH5, named tentatively as the bacteriocin produced by *Lactococcus lactis* BH5, was detected at the mid-log growth phase, reached its maximum during the early stationary phase, and decreased after the late stationary phase. Lacticin BH5 also showed a relatively broad spectrum of activity against non-pathogenic and pathogenic microorganisms as tested by the spot-on-lawn method. Its antimicrobial activity on sensitive indicator cells was completely disappeared by protease XIV or α-chymotrypsin. The inhibitory activities of lacticin BH5 were detected during treatments up to 100℃ for 30 min. Lacticin BH5 was very stable over a pH range of 2.0 to 9.0 and was stable with all the organic solvents examined. The cell concentration and bacteriocin production in strain BH5 were maximum when grown at 30°C in a modified MRS medium supplemented with 0.5% tryptone, 1.0% yeast extract, and 0.5% beef extract as nitrogen sources. It demonstrated a typical bactericidal mode of inhibition against *Micrococcus flavus* ATCC 10240. Lacticin BH5 was purified through ammonium sulfate precipitation, ethanol precipitation, and CM-Sepharose column chromatography. The apparent molecular mass of lacticin BH5 was estimated to be in the region of 3.7 kDa, by the direct detection of bactericidal activity after SDS-PAGE. Mutant strain NO141 which was isolated by nitrosoguanidine mutagenesis produced about 4 fold more bacteriocin than the wild type.

Introduction

Lactic acid bacteria are common microflora in various fermented foods such as dairy products and processed vegetables, and play an essential role in food fermentation processes. They have been used for centuries in the preparation and preservation of foods based on meat, milk, and of vegetable origin and are generally recognized as safe (GRAS) (1, 2). Many lactic acid bacteria produce antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, carbon dioxide, and bacteriocins (1).

Bacteriocins are defined as bactericidal proteins, which generally have a narrow spectrum of activity targeted toward a species related to the producers culture (3). Recently, bacteriocins have aroused great interest in the context of food preservation. The possibility of genetically manipulating the genes encoding bacteriocins is considered as one of the major reasons for undertaking bacteriocin research (4, 5). Bacteriocin producers have developed a protection system against their own bacteriocin, this system is referred to as self-immunity and enables them to be unaffected by their own bacteriocin. Bacteriocins are potentially useful for industrial application because of their antibacterial activities, moreover, the bacteriocin can be used as a biopreservative and bioregulator of the microflora present in fermented-food (6). Nisin, a GRAS bacteriocin, is produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is the only bacteriocin which has been approved for food use in many countries (7). It is believed that further research will allow of other bacteriocins to be successfully

exploited as food preservatives.

Kimchi is a traditional Korean fermented food, which is prepared by a series of processes, which include the pretreatment of oriental cabbages, brining, blending with various spices and other ingredients, and fermentation. The species of lactic acid bacteria, which becomes predominant during Kimchi fermentation can influence the storage and quality. Kimchi is a unique source of lactic acid bacteria, especially as it originates from vegetables and fermented fish sauces. Despite, the realized importance and industrial value of bacteriocins in Korea they have been underestimated. However, many Kimchi bacteriocins were identified and characterized when compared with bacteriocins from other sources (8-12).

In this study we report on the enhanced production, purification, and partial characterization of lacticin BH5, a Kimchi bacteriocin produced by *Lactococcus lactis* BH5. The characteristics of lacticin BH5 make this bacteriocin potentially interesting as an antimicrobial agent for the control of both spoilage and pathogenic organisms in foods.

Identification of a lacticin BH5 producer

Strain BH5 was isolated from naturally fermented Kimchi and identified as a bacteriocin producer. The bacteriocin-producing strain BH5 was identified by Gram staining, morphology by SEM (Fig. 1), and from biochemical carbohydrate fermentation patterns (Table 1) and physicochemical characteristics (Table 2). On the basis of these results this strain was identified as *Lacococcus lactis*, and the isolate was tentatively named as *Lactococcus lactis* BH5. Lacticin BH5 is proposed as the tentative name of the bacteriocin produced by *L. lactis* BH5.

Production of lacticin BH5

In MRS medium of pH 6.0 and incubated at 30℃, *L. lactis* BH5 produced extracellular inhibitory activity against *M. flavus* ATCC 10240 (Fig. 2). Production of lacticin BH5 seems to follow the kinetics typical of primary metabolite synthesis. Lacticin BH5 activity reached a maximum (12,800 AU/ml) after incubation for 5 h, the early stationary phase, and was maintained for 1 h before dropping after the late stationary phase. Possible reasons for this rapid decrease in bacteriocin activity include, the formation of an inhibitor, its degradation by extracellular proteolytic enzymes, the binding of bacteriocin to cells, and inactivating complex formation with other extracellular products (13). Almost all the bacteriocins of lactic acid bacteria are produced during the exponential growth phase. Several lantibiotics are also synthesized during the exponential phase of the producer strain. This comparatively early bacteriocin production (5 h) by *L. lactis* BH5 could be exploited as an industrially useful characteristic.

Medium optimization for enhanced bacteriocin production was also studied (data not shown).

Antimicrobial spectrum of activity

To determine the antimicrobial spectrum of activity, the cell-free supernatant and partially purified lacticin BH5 were tested against various non-pathogenic and pathogenic bacteria, a yeast and molds using the modified deferred and spot-on-lawn methods (Table 3).

L. lactis BH5 showed broad spectrum of activity against all of the non-pathogenic and pathogenic bacteria tested by the modified deferred method, but did not show antimicrobial activity against a yeast and molds. Lacticin BH5 also showed a relatively broad spectrum of activity against most of lactic acid bacteria, *Staphylococcus aureus* KCCM 32359, *Staphylococcus epidermidis* ATCC 12228, *Clostridium perfringens* ATCC 3624, some bacilli, *Micrococcus flavus* ATCC 10240, *Listeria monocytogenes* ATCC 15313, *Listeria ivanovii* ATCC 19119, *Yersinia enterocolitica* ATCC 27729, *Escherichia coli* KCCM 32396, and *Pseudomonas fluorescens* using the spot-on-lawn method, when compared with other bacteriocins of lactic acid bacteria. However, inhibitory activity was not observed against a yeast and molds. Accordingly, from its inhibitory spectrum, lacticin BH5 appeared to show

similarity with lantibiotic nisin, which inhibits most Gram-positive bacteria (14), rather than several bacteriocins from *Lactobacillus* sp., whose activity spectrums include only strains, which belong to the same genus (15-17).

Effects of various enzymes, heat, pH, and organic solvents

As shown in Table 4, treatment with protease XIV or α-chymotrypsin caused a complete loss of bacteriocin activity. No modification of activity was observed when lacticin BH5 was treated with the other enzymes tested (protease IX, pepsin, proteinase K, α-amylase, and lipase). Buffers and enzyme solutions alone had no effect on the indicator strain. When the partially purified bacteriocin was treated with lipase and amylases, the bacteriocin activity was not changed. These results confirm the proteinaceous nature of the antimicrobial substance and suggest that neither lipid nor carbohydrate moieties are essential for the bacteriocin activity.

Lacticin BH5 proved to be relatively heat stable (Table 4); partially purified lacticin BH5 was stable to heat treament at 90℃ for 30 min. Inhibitory activities were detected during treatment up to 100℃ for 30 min, however, the inhibitory activity of partially purified lacticin BH5 was inactivated by heat at 121℃ for 15 min. This heat stability could be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions, stable cross-linkage, and a high glycine content (18). This heat stability also rules out the possibility that the inhibitory action is due to bacteriophage.

Finally, partially purified bacteriocin was pH stable in the range 2.0 to 9.0 and it was not affected by any of the organic solvents shown in Table 5. This aspect of its stability is of particular importance to the food industry.

Mode of Inhibition

To determine whether lacticin BH5 has a bactericidal or a bacteriostatic effect, the partially purified lacticin BH5 was added to the indicator cells suspended in phosphate buffer (pH 7.0). Lacticin BH5 showed a bactericidal mode of action. A decrease in CFU per milliliter was observed after the bacteriocin was exposed to the indicator cells (Fig. 3). However, the intrinsic nature of this inhibition has not been identified and requires further investigation.

Purification

Lacticin BH5 was purified completely through ammonium sulfate precipitation, ethanol precipitation, and CM-Sepharose column chromatography. After passage through the column, 91.4% of the bacteriocin activity was recovered and the specific activity was increased 263.7 fold (Table 5).

Molecular weight of lacticin BH5

The polyacrylamide gel, containing purified lacticin BH5, was cut into two vertical sections. The gel portion containing the sample and the molecular weight markers was stained, while the remaining section, which contained only the sample, was fixed and used for the direct detection of antimicrobial activity. As shown in Fig. 4, the bactericidal activity of lacticin BH5 was associated with a band having an apparent molecular mass of 3.7 kDa. Thus, the apparent molecular mass of lacticin BH5 was determined to be 3.7 kDa by the direct detection of bactericidal activity after SDS-PAGE.

Selection of over-producing mutants

Three over-producing mutants were isolated by NTG mutagenesis and their bacteriocin production was compared with parent strain. Among them, mutant strain NO141 produced about 4 fold more bacteriocin than the wild type in flask fermentation (Table 6). Lacticin BH5 activities

produced by *L. lactis* BH5 and mutant NO141 were 8,192 AU/ml and 32,768 AU/ml, respectively, when they were grown in a 5L jar fermenter (data not shown).

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Table 1. Microbiological identification of strain BH5 using its carbon source utilization pattern.

Carbohydrate	Isolate BH5	Carbohydrate	Isolate BH5	Carbohydrate	Isolate BH5
Glycerol	-[a]	Inositol	-	Inuline	-
Erythritol	-	Mannitol	+	Melezitose	-
D-Arabinose	-	Sorbitol	-	D-Raffinose	-
L-Arabinose	+	α-Methyl-D-mannoside	-	Amidon	+
Ribose	+	α-Methyl-D-glucoside	-	Glycogen	-
D-Xylose	+	N-Acetyl glucosamine	+	Xylitol	-
L-Xylose	-	Amygdaline	+	β-Gentibiose	+
Adonitol	-	Arbutine	+	D-Turanose	-
β-Methyl-xyloside	-	Esculine	+	D-Lyxose	+
Galactose	+	Salicine	+	D-Tagatose	-
D-Glucose	+	Cellobiose	+	D-Fucose	-
D-Fructose	+	Maltose	+	L-Fucose	-
D-Mannose	+	Lactose	+	D-Arabitol	-
L-Sorbose	-	Melibiose	-	L-Arabitol	-
Rhamnose	-	Saccharose	+	Gluconate	+
Dulcitol	-	Trehalose	+	2-cetogluconate	-
				5-cetogluconate	-

[a] Data obtained using a API 50 CHL kit. + : positive, - : negative.

Table 2. General and physicochemical characteristics of strain BH5.

Chracteristics	Results
Morphology	Cocci
Gram staining	+
Oxygen requirement	±
Catalase	-
Growth in/at	-
6.5% NaCl	-
45℃	-
Acetoin production	+
Hippurate hydrolysis	-
Production of :	-
β-glucosidase	(+)
Pyrrolidonylarylamidase	-
α-galactosidase	-
β-glucuronidase	-
β-galactosidase	-
alkaline phosphatase	-
leucine arylamidase	+
arginine dihydrolase	+
Fermentation of catbohydrates	-
ribose	+
L-arabinose	+
mannitol	+
sorbitol	-
lactose	+
trehalose	+
inulin	-
raffinose	-
starch	-
glycogen	-
Hemolysis	-
Spore-forming	-

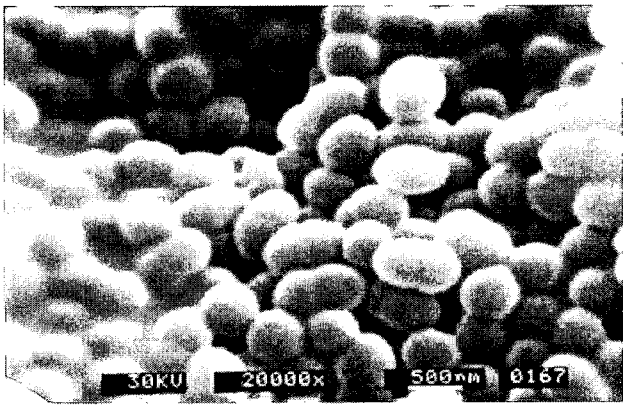

Figure 1. Scanning electron microscopic (SEM) observation of strain BH5.

Table 3. Antimicrobial spectrum of activity of partially purified lacticin BH5 using the spot-on-lawn method.

Organisms	Culture medium[b]	Incubation temp.	Inhibition	
			Modified deferred method	Spot-on-lawn method
Gram positive bacteria				
Bacillus cereus ATCC 11778	TSB	37°C	12.0	+
Bacillus cereus	NB	30°C	16.0	+
Bacillus pumilis	NB	30°C	30.0	+
Bacillus subtilis ATCC 6633	TSB	37°C	4.0	+/-
Bacillus subtilis IFO 12113	LB	37°C	22.0	-
Clostridium perfringens ATCC 3624[a]	TSB	37°C	32.0	+
Corynebacterium xerosis NCTC 9755	TSB	37°C	6.5	-
Enterococcus faecalis ATCC 19433	TSB	37°C	8.0	+/-
Lactobacillus delbrueckii ATCC 4797	MRS	37°C	23.5	+
Leuconostoc curvatus CA170-12	MRS	25°C	19.0	+
Leuconostoc mesenteroides KCCM 11324	MRS	25°C	14.0	+
Listeria ivanovii ATCC 19119	TSB	30°C	20.5	+
Listeria monocytogenes ATCC 15313	TSB	30°C	26.0	+
Micrococcus flavus ATCC 10240	NB	30°C	25.0	+
Pediococcus acidilactici KCTC 1626	MRS	37°C	15.5	+
Staphylococcus aureus ATCC 25923	TSB	37°C	15.0	+/-
Staphylococcus aureus KCCM 32359	NB	37°C	21.5	+
Staphylococcus epidermidis ATCC 12228	TSB	37°C	16.5	+/-
Gram negative bacteria				
Escherichia coli JM109	LB	37°C	22.0	-
Escherichia coli KCCM 32396	LB	37°C	26.0	+
Escherichia coli O157:H7	TSB	37°C	9.5	-
Pseudomonas aeruginosa ATCC 15442	TSB	30°C	9.0	-
Pseudomonas fluorescens	NB	30°C	18.0	+
Pseudomonas putida	NB	30°C	>40.0	-
Pseudomonas syringae ATCC 12885	TSB	30°C	7.0	-
Salmonella enteritidis	TSB	37°C	10.0	-
Salmonella paratyphi	TSB	37°C	16.0	-
Salmonella typhi	TSB	37°C	14.5	-
Samonella typhimurium	TSB	37°C	13.5	-
Shigella boydii	TSB	37°C	11.5	-
Shigella flexeri	TSB	37°C	13.5	-
Shigella sonnei	TSB	37°C	11.5	-
Vibrio cholerae non-O1	TSB	37°C	7.5	+/-
Vibrio cholerae O 139	TSB	37°C	20.5	ND
Vibrio parahaemolyticus ATCC 17802	TSB	37°C	8.0	+/-
Vibrio vulnificus	TSB	37°C	23.5	+/-
Yersinia enterocolitica ATCC 27729	TSB	30°C	28.0	+
Yeasts and Molds				
Aspergillus oryzae KCCM 11371	PDB	30°C	0	-
Aspergillus niger KCCM 11239	PDB	30°C	0	-
Penicillium chrysogenum KCCM 6933	PDB	30°C	0	-
Saccharomyces cerevisiae KCCM 11201	YPD	30°C	0	-

[a] Incubated in anaerobic GasPak jar.

[b] NB, nutrient broth; LB, Luria broth; TSB, tryptic soy broth; MRS, lactobacilli MRS broth; PDB, potato dextrose broth; YPD, yeast extract peptone dextrose.

[c] Not clear.

[d] Not determined.

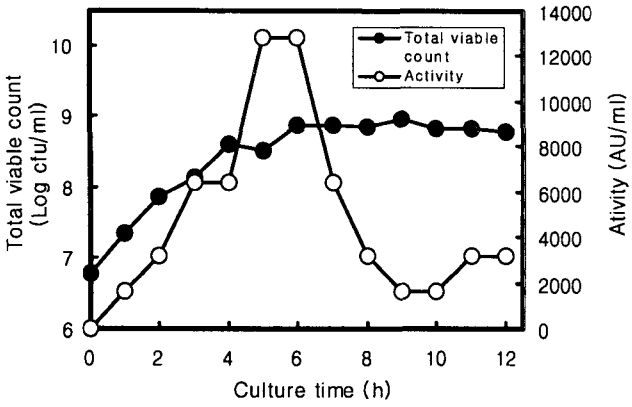

Figure 2. Production of lacticin BH5 in jar fermenter.

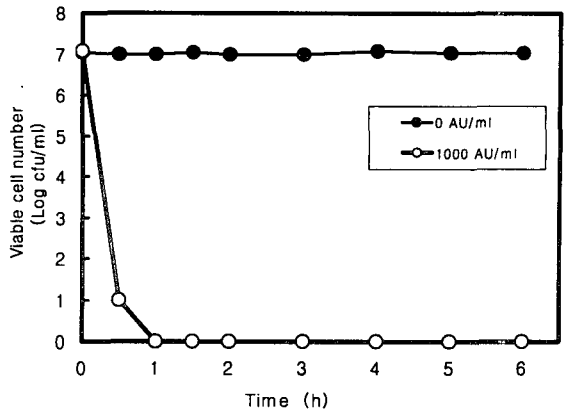

Figure 3. Mode of action of lacticin BH5 against *M. flavus* ATCC 10240 in phosphate buffer.

Table 4. Effect of various enzymes, pH, heat, and organic solvents on partially purified lacticin BH5.

Treatment	Residual activity (AU/ml)	Treatment	Residual activity (AU/ml)	Treatment	Residual activity (AU/ml)	Treatment	Residual activity (AU/ml)
Organic solvent		**Enzyme**		**pH**		**Heat**	
Control	51,200	Control	51,200	Control	51,200	Control	102,400
Ethanol	51,200	Protease XIV	0	2.0	51,200	40℃[a]	102,400
Methanol	51,200	Protease IX	51,200	3.0	51,200	50℃[a]	102,400
Acetone	51,200	Pepsin	51,200	4.0	51,200	60℃[a]	102,400
Toluene	51,200	α-Chymotrypsin	1,600	5.0	51,200	70℃[a]	102,400
Isopropyl	51,200	Proteinase K	51,200	6.0	51,200	80℃[a]	102,400
chloroform	51,200	α-Amylase	51,200	7.0	51,200	90℃[a]	102,400
		Lipase	51,200	8.0	51,200	100℃[a]	12,800
				9.0	51,200	121℃[b]	0

[a]Heat treatment for 30 min.
[b]Autoclave for 15 min.

Table 5. Summary of purification for lacticin BH5.

Purification step	Total activity (AU)	Total protein (mg)	Specific activity (AU/mg)	Fold	Yield (%)
Culture fluid	16384000.0	21731.3	753.9	1.0	100.0
Ammonium sulfate precipitation	16384000.0	2325.4	7045.7	9.3	100.0
Ethanol precipitation	15073280.0	201.5	74805.4	99.2	92.0
Ion exchange CM-Sepharose	14968422.4	75.3	198783.0	263.7	91.4

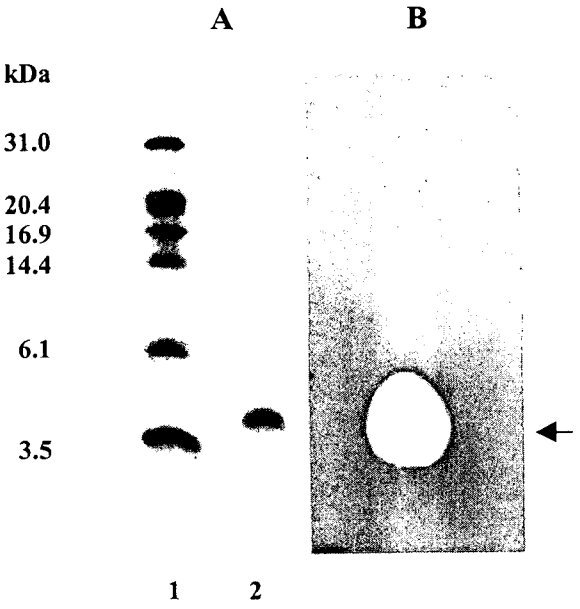

Fig. 4. Tricine-SDS-PAGE of purified lacticin BH5.
Lane 1, Low molecular weight standard:
Lane 2, Purified bactericin.

Table 6. Comparison of bacteriocin activity in *Lactococcus lactis* BH5 and mutant strains.[a]

Strains	[b]Cell conc. (OD_{660})	[c]Bacteriocin activity (AU/mℓ)
BH5	2.9	4096
Mutant JU 61-1	3.0	8192
Mutant JU 73-2	2.9	8192
Mutant NO141	2.7	16384

[a]Cell was grown in a 500ml-Erlenmeyer flask containing 50ml of TBY medium at 30℃.
[b]Cell concentration and [c]bacteriocin activity were measured at time intervals and expressed as the maximum value.