Journal of Life Science **2004**, Vol. 14. No. 4. 683~688 ©JLS

Characterization of Bacteriocin, lacticin YH-10, Produced by *Lactococcus lactis* subsp. *lactis* YH-10 Isolated from Kimchi

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Received July 13, 2004 / Accepted August 11, 2004

A bacteriocin-producing lactic acid bacteria was isolated from Kimchi on MRS selective media with the use of *Lactobacillus delbrueckii* subsp. *delbrueckii* as an indicator strain. The strain YH-10 was identified as *Lactococcus lactis* subsp. *lactis* through the API test. The crude bacteriocin (freeze-dried 50% ammonium sulfate precipitate of culture supernatant) produced by the strain was named as lacticin YH-10. Lacticin YH-10 showed the growth inhibitory activity against Gram positive pathogenic bacteria and other lactic acid bacteria. The bacteriocin was inactivated by proteases such as protamex and aroase AP-10 and partially inactivated by amylase, proteinase K, trypsin, and papain. The lacticin YH-10 remained its activity with the treatment of heat at 100℃ for 60 min or the changes of pH 2 to 11. However, the activity was lost at high pH combined with the exposure to 100℃. The bacteriocin production of the strain was started in the exponential phase and stopped in the stationary phase. The approximate molecular mass of the bacteriocin produced by the strain was approximate 14 kDa in the analysis on SDS-PAGE.

Key words – Bacteriocin, *Lactococcus lactis* subsp. *lactis*, lacticin YH-10, Kimchi

Lactic acid bacteria (LAB) are important in food fermentation because they are used as starter cultures in most food fermentation and also have antimicrobial activity attributed to the production of bacteriocins [5,8,11,14,18,20]. Bacteriocins are peptides or proteins that show a bactericidal action against the bacteria closely related to the producer strain. Their proteinous nature implies enzymatic degradation in the gastrointestinal tracts of human and animals. Thus, bacteriocins produced by starter cultures are excellent candidates for the improvement of the safety of various fermented foods [22].

Interest in these compounds from LAB is led to the commercial use of the antibiotic nisin, which has been the most extensively studied bacteriocin from the genus *Lactococci* and approved for use as a food preservative [17].

The screening of new bacteriocin in Korea is promising because of the relative abundance of traditionally fermented foods such as Jang (fermented soybean sauce and paste), Kimchi (fermented vegetables), and Jeot-gal (fermented fish food). The studies on the bacteriocin and lactic acid bacteria isolated from Kimchi have been reported [2,4,10,15,16,19]. Various microorganisms originally present in the raw materials initiate their growth during Kimchi fermentation process, but lactic acid bacteria gradually become dominant species with producing bacteriocin and other metabolites [14].

In this study, a strain producing bacteriocin in Kimchi fermentation was identified and the properties of the bacteriocin, lacticin YH-10, were characterized.

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Materials and Methods

Bacterial Strains and Media

A LAB YH-10 was isolated from fermented Kimchi. The isolated LAB YH-10 was identified as *Lactococcus lactis* subsp. *lactis* on the basis of Gram staining, the catalase test, morphology with SEM (Scanning electron microscopy), its carbohydrates fermentation patterns and biochemical characteristics through API 50CHL system (BioMerieux, France). *L. lactis* subsp. *lactis* YH-10 was cultured for 10 h at 25℃ either in MRS broth (Difco laboratories, Detroit, U.S.A.) or on the equivalent solid media prepared by adding 1.5% agar. The strain was stored at -70℃ in MRS broth containing 33.3% glycerol.

Bacteriocin Activity Assay

MRS medium was sterilized by autoclaving (15 min, 121 ℃) and 1% (vol/vol) of a 10 h culture of *L. lactis* subsp. *lactis* YH-10 was inoculated to the medium. Its initial pH was 6.5. The temperature was held at 25℃. Samples were

removed at different time intervals for the determination of cell mass at 660 nm of absorbance and the bacteriocin activities.

Bacteriocin was detected by the agar well diffusion method and its activity was measured by a serial dilution method [21]. For the agar-well assay, the cell-free supernatant broth was placed into the wells cut on agar plate previously inoculated with *Lactobacillus delbruekii* subsp. *delbrukii* as indicator strain. Wells (6 mm) were filled with 50 μl of test samples. The plate was left for 4 h at 4℃ and then incubated at the optimum growth temperature of the indicator strain. After 24 h, the plate was examined for zones of inhibition.

Activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed as activity units (AU) per milliliter [7]. Assays were performed in triplicate.

Characterization of Bacteriocin

Sensitivity to various enzymes was tested by treating lacticin YH-10 with α-amylase, β-amylase, glucoamylse, catalase, glutaminase, aroase AP-10, protamex, proteinase K, trypsin, and papain. All enzymes were suspended in buffers as recommended by suppliers, and the substance was mixed with equal volumes of enzyme suspensions and incubated for 2 h at 37℃. For each of the enzymes tested, controls were incubated without bacteriocin. Residual activity was determined by the agar well diffusion assay. To evaluate heat stability, aliquot of lacticin YH-10 was heated at 100℃ for 10, 30 and 60 min and at 121℃ for 15 min. The pH stability of lacticin YH-10 was estimated after 4 h storage at 4℃ in the following buffers, 50 mM citrate buffer at pH 2-5, 50 mM phosphate buffer at pH 6-7, 50 mM Tris-HCl buffer at pH 8-9 and 50 mM sodium bicarbonate at pH 10-11 [23]. Also, dual effect with temperature and pH for lacticin YH-10 was determined. The pH of bacteriocin was adjusted to different values by either HCl or NaOH and then the samples were placed in a water bath at 100℃. Aliquot (500 μl) was removed after 10, 30, 60 and 120 min and their bacteriocin activities were determined by the serial dilution method.

Mode of Action

Cells at the end of exponential phase in the growth of *Lactobacillus delbruekii* subsp. *delbrukii* as the indicator strain were suspended in sterile MRS broth [13]. The test was carried out at 37℃ by the addition of 40 AU or 320 AU of lacticin YH-10 to the cultures of the indicator strain without incubation or after 2 h of the incubation. The sample was removed at appropriate times for the absorbance measurement at 660 nm and viable count on MRS agar plate.

Partial Purification of the Substance Produced by *L. lactis* subsp. *lactis* YH-10

The substance was partially purified using an ammonium sulfate precipitation method. The culture broth was centrifuged at 708×g for 30 min at 4℃ (Beckman Coulter, Inc., U.S.A.). The supernatant was adjusted to pH 6.5 to avoid the isoelectric point, and it was then boiled for 10 min to inactivate protease [3,15]. The cell-free suprenatant was transferred to a beaker in an ice bath, and ammonium sulfate was added at 40% final concentration. Subsequently, the mixture was centrifuged at 708×g for 30 min at 4℃ (Beckman Coulter, Inc., U.S.A.) and the pellet was then dissolved in 50 mM potassium phosphate buffer of pH 6.5. This solution was dialyzed against the same buffer overnight using a membrane with 3.5 kDa cutoff (Spectrum Medical Inc., LA, U.S.A.) [12]. The dialyzed material (crude substance) was freeze-dried and then stored at -20℃ until use.

Determination of Molecular Weight by SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 16.5% acrylamide gel as described previously [1]. A 15 μl aliquot of the 20 fold concentrated bacteriocin sample was mixed with 15 μl 2 fold concentrated sample buffer and boiled for 5 min. After electrophoresis, the gel was divided into two parts. The first half, with standards and bacteriocin samples, was stained with Coomassie R250 stain. The other part containing only bacteriocin samples was used for activity detection by a direct detection method [6]. The unstained half was fixed with 40% methanol-10% acetic acid-10% glutaraldehyde for 1 h and washed in deionized water, with frequent exchange of the water for 2 h. The washed gel was overlaid with the indicator strain *Lactobacillus delbruekii* subsp. *delbreukii*. After incubation, the position of the inhibition zone was detected and compared to the stained gel part.

Results and Discussion

Classification of Strain YH-10

The phenotype characteristics of strain YH-10 were

tested through Gram staining and catalase activity. The physiological characteristics were examined by an API 50 CHL kit (BioMerieux, France). The bacterial cells were Gram-positive, catalase-negative and a coccus type. The carbohydrate utilization patterns of strain YH-10 coincided with those of *Lactococcus lactis* subsp. *lactis*. Therefore, the strain was named as *Lactococcus lactis* subsp. *lactis* YH-10. The bacteriocin produced from this strain was named as lacticin YH-10.

Spectrum of Antimicrobacterial Activity

The inhibitory activity of lacticin YH-10 prepared by ammonium sulfate precipitation was tested against various Gram-positive and Gram-negative bacteria. The results are shown in Table 1. The antimicrobial spectrum of lacticin YH-10 was broad, effective not only against LAB including genus *Lactobacillus*, *Pediococcus* and *Leuconostoc* but also against Gram positive bacteria such as *Acetobacter* and *Pseudomonas*. Especially, lacticin YH-10 showed inhibitory activity for spore forming *Bacillus subtilis* and *Acetobacter aceti*. It seems that the inhibition of *Acetobacter sp.* by the bacteriocin can contribute the flavor maintenance of Kimchi during its long storage.

Effects of Temperature and pH on the Production of Lacticin YH-10

The optimal temperatures for cell growth and bacteriocin production were determined as shown in Fig. 1. *Lactococcus lactis* subsp. *lactis* YH-10 showed that the maximum

Table 1. Inhibitory spectrums of lacticin YH-10

Indicator strain	The inhibition diameter of halo (mm)
Lactobacillus helveticus CNRZ1096	10
Lactobacillus helveticus CNRZ1094	20
Lactobacillus plantarum ATCC1083	8
Lactobacillus brevis IFO13109	9
Lactobacillus casei KCRZ1121	8
Lactobacillus delbrueckii IFO3534	10
Lactobacillus fermentum	10
Leuconostoc mesenteroides ATCC10830	8
Bacillus subtillus 168	6
Bacillus subtillus 3111	10
Streptococcus mutans ATCC25922	4
Pediococcus acidilactis KCTC3101	9
Escherichia coli ATCC9637	-
Listeria monocytogens	-
Pseudomonas synsantha	12
Acetobacter aceti	14

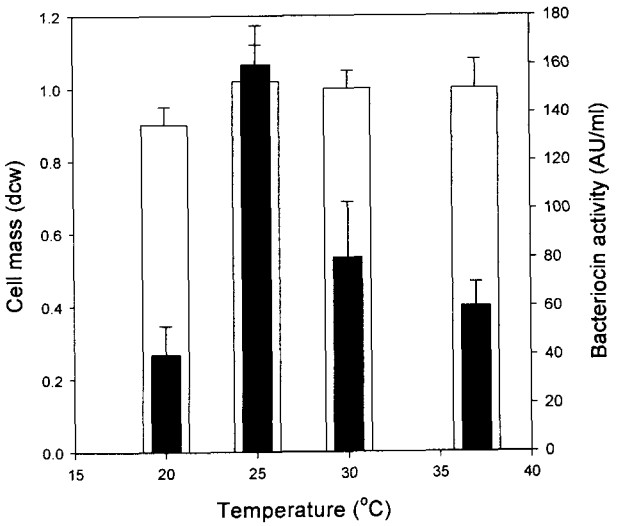

Fig. 1. Effect of temperature on the cell growth and bacteriocin production of *Lactococcus lactis* subsp. *lactis* YH-10. Legends: (□) maximum cell mass (dcw); (■) maximum bacteriocin activity (AU/ml).

bacteriocin activity was obtained at 25℃ while the cell growth was almost same from 25 to 37℃. Lacticin YH-10 was produced during the exponential growth phase at 25 ℃ (Fig. 2). Usually, bacteriocin producing strain from Kimchi has a trend of the maximum production of bacteriocin at low temperature. This can explain the flavor development of Kimchi at low temperature.

The activity reached a maximum 160 (AU/ml) at 10 h

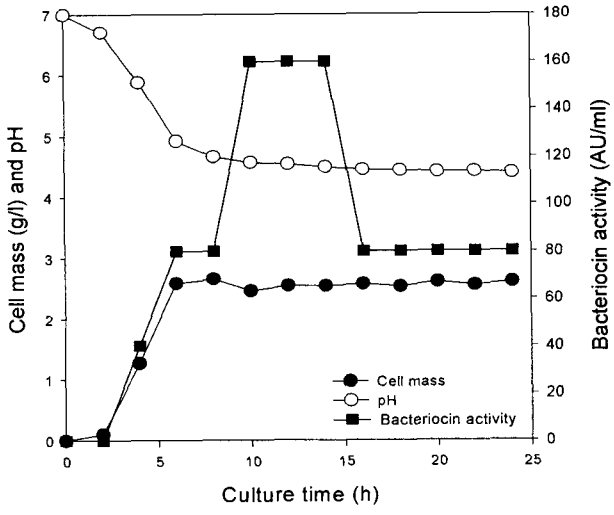

Fig. 2. The curve of bacteriocin production from *Lactococcus lactis* subsp. *lactis* YH-10 at 25℃ with 7 as the initial pH.
The time-dependent change of bacteriocin activity was monitored together with the change in pH and cell density.

of the culture. After 10 h of incubation, the maximum activity of the lacticin YH-10 was maintained for 4 h as shown in Fig. 2. *Lactococcus lactis* subsp. *lactis* YH-10 produced bacteriocin in direct proportion to growth curve. Therefore, it was assumed that this strain in Kimchi fermentation dominates microbial flora and, thus, enhances the flavor of Kimchi.

Characterization of Lacticin YH-10

Lacticin YH-10 showed thermal stability as seen in other typical bacteriocin. Its activity was not affected by the heat treatment at 100℃ for 30 min and detected even after the heat treatment at 100℃ for 60 min (Table 2). The effect of various enzyme treatments on the lacticin YH-10 was evaluated as shown in Table 2. The bacteriocin activity of lacticin YH-10 was reduced by treatments with α-amylase, β-amylase, glucoamylase, catalase, proteinase K, trypsin and papain, and completely removed by aroase AP-10 and protamex of *Bacillus* protease, indicating the proteineous characteristics of a bacteriocin. The reduction of the antimicrobial activity of lacticin YH-10 by α-amylase, β-amylase and glucoamylase suggests that carbohydrate moieties are associated with the antimicrobial activity. This may indicates that the lacticin YH-10 can be grouped into class Ⅳ bacteriocin [8].

Lacticin YH-10 prepared by ammonium sulfate precipitation was stable within a wide pH range from 2 to 11 (Fig. 3). Measurement of antibacterial activity at 100℃ over the pH range from 2 to 10 indicated that lacticin YH-10 was

Table 2. Effect of heat and enzyme treatment on the activity of lacticin YH-10

Residual activity (AU/ml)	Treatment	
	640 Control (untreated)	
Heat	100℃, 10 min	640
	100℃, 30 min	640
	100℃, 60 min	320
	121℃, 15 min	0
Enzymes	α-amylase	160
	β-amylase	160
	Glucoamylase	160
	Catalase	160
	Glutaminase	160
	Aroase AP-10	0
	Protamex	0
	Proteinase K	40
	Trypsin	160
	Papain	160

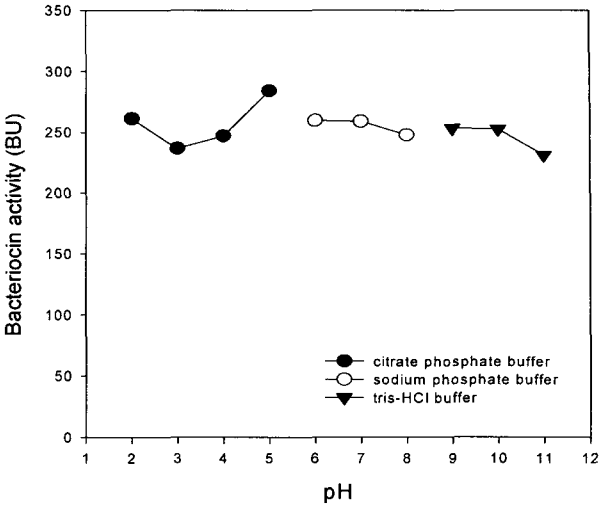

Fig. 3. Effect of pH on the activity of lacticin YH-10 solution were adjusted by 50 mM citrate-phosphate buffer (pH 2-5), 50 mM sodium phosphate buffer (pH 6-8), and 50 mM Tris-HCl buffer (pH 9-11).

more stable at low pH values at the elevated temperature comparing to other pH ranges (Fig. 4). These data suggest that the bacteriocin could be a useful preservative in the preservation of acidic foodstuffs, such as fermented products which require heat treatment for a long-term storage.

Mode of Action

To evaluate the mode of the antibiotic action of lacticin YH-10, the action mode of lacticin YH-10 on the viability and the lysis of indicator cells was examined. *Lactobacillus delbruekii* subsp. *delbruekii* was incubated with lacticin

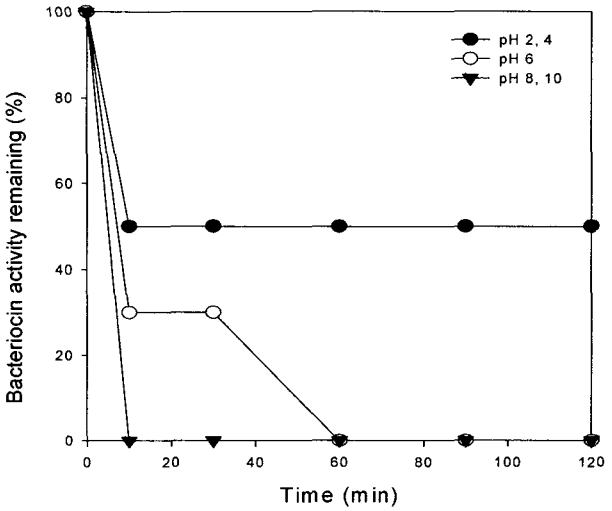

Fig. 4. Time of x-axis indicates effect of pH on the bactericidal activity of lacticin YH-10 at 100℃.

YH-10 (640 AU/ml) (Fig. 5). Three hours of incubation of indicator cells with the bacteriocin resulted in 99% cell death as determined by colony forming unit (CFU). However, the optical density remained constant throughout the experiment. These results indicate that lacticin YH-10 acts bactericidal mode rather than bacteriolytic mode to sensitive cells.

Determination of Molecular Weight by SDS-PAGE

After SDS-PAGE, the bacteriocin band was determined by an inhibition zone with the soft agar overlay method. The clear band was observed at the position of 14 kDa, as determined from the comparison with the low range molecular mass marker (BIO-RAD) (Fig. 6). The band from coomassi blue staining could be detected in lane B at corresponding position to the clear bands at lane C1 and C2.

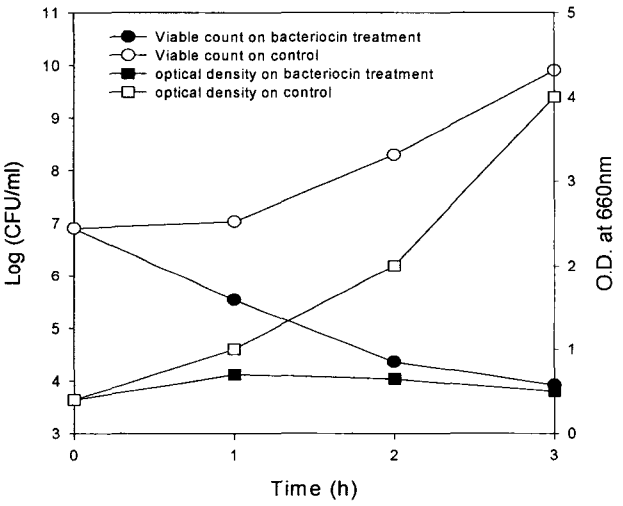

Fig. 5. Bactericidal effect of lacticin YH-10 against *Lactobacillus delbruekii* subsp. *delbruekii* from log-phase (640 AU/ml).

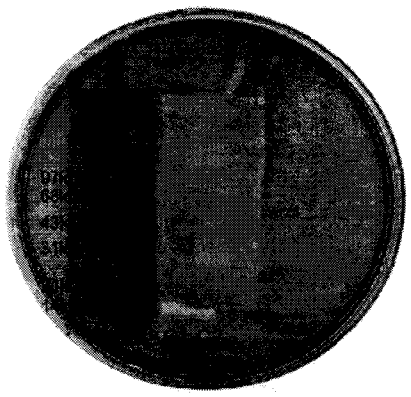

Fig. 6. SDS-PAGE and antimicrobial activity of lacticin YH-10. A; standard marker, B; lacticin YH-10 stained with coomassie blue R-250 reagent, C1 and C2; antimicrobial activity with *Lactobacillus delbruekii* subsp. *delbruekii* as an indicator strain.

Acknowledgment

This work was supported by 04' Fundfrom Research Center of Industry-Academy co-operation. Eun-Min Choi was supported by Brain Korea 21 and So-Jin Park was supported by Brain Busan 21 project.

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초록 : 김치유산균인 *Lactococcus lactis* subsp. *lactis* YH-10가 생산하는 박테리오신의 특성

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API test를 통해서 김치에서 분리한 유산균이 *Lactococcus lactis* subsp. *lactis*의 한 종임을 확인하고 *Lactococcus lactis* subsp. *lactis* YH-10이라 명명하고, 생산된 박테리오신을 lacticin YH-10으로 명명하였다. *Lactococcus lactis* subsp. *lactis* YH-10을 25℃, 30℃와 37℃에서 배양한 결과 25℃에서 박테리오신을 생성하였다. Lacticin YH-10은 여러 가지 단백질 분해효소에 의해 항균활성을 소실하였으므로, 단백질로된 박테리오신임을 확인하였다. 또한 α-amylase, β-amylase, glucoamylase의 처리에도 활성을 일부 소실하였으므로 박테리오신의 분류 중 classⅣ에 속함을 알 수 있었다. Ammonium sulfate 정제한 후 SDS-PAGE를 통해 박테리오신의 분자량이 대략 14kDa임이 확인되었다. Lacticin YH-10은 단백질계 물질로서 인체 내에서 분해가 가능하기 때문에 일반 화학 보존제 보다 인체에 안정함을 알 수 있다. Lacticin YH-10은 낮은 pH와 높은 온도에서 항균활성을 가지고 있기 때문에 식품보존제로서 이용할 수 있다.