Characterization of Bacteriocins produced by Lactic Acid Bacteria against bovine mastitis pathogens

젖소 유방염 유발균주를 저해하는 Lactic acid bacteria에서 생산되는 ba[illegible]에 관한 연구

Adv[illegible]

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A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Engineering

in the Department of Biotechnology and Bioengineering, Graduate School,
Pukyong National University

February 2004

Characterization of Bacteriocins produced by Latic Acid Bacteria against bovine mastitis pathogens

A Dissertation

by

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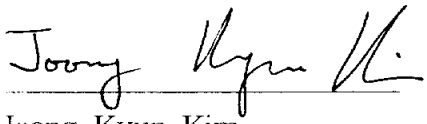

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December 25, 2003

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요 약

젖소 유방염은 우유의 질적 양적으로 감소를 유발시켜 낙농업계에서 심각한 질병으로 여겨지고 있다. 젖소 유방염의 발생으로 인해 원유의 생산량이 감소하는 할 뿐 아니라 버려지는 원유의 양의 증가로 인해 커다란 경제적 손실을 주고 있다. *Staphylococcus aureus*와 *Streptococcus agalactiae*에 의한 젖소 유방염의 발생이 전체 유방염의 발생의 70%를 차지하고 있다. 이러한 젖소 유방염의 치료를 위해 주로 항생제가 사용되고 있다. 하지만 항생제의 사용은 원유 내에 항생제 잔류의 영향으로 항생제 치료 후 일정기간동안 원유의 판매가 금지되고 있다. 이런 문제에 대한 대체 방안으로 단백질성 물질인 bactericins를 이용하고자 연구를 수행하였다.

Bacteriocin을 생산하는 균주로 *Lactobacillus bulgaricus*와 *Lactococcus lactis*를 MRS배지를 사용하였으며 indicator strains로는 *Staphylococcus aureus*와 *Streptococcus agalactiae*를 사용하였다. *L. bulgaricus*와 *L. lactis*에서 생산되는 bacteriocin은 gram-positive bacteria와 gram-negative bacteria에 대해 넓은 항균활성을 나타내었다. 특히 젖소 유방염의 주요 균주인 *Staph. aureus*와 *Strep. agalactiae*에 대해서도 항균활성을 가짐을 알 수 있었다. *L. bulgaricus*에서 생산되는 bacteriocin을 bulgaricin HJ로 *L. lactis*에서 생산되는 bacteriocin을 lacticin HJ로 명명하였다.

*L. bulgaricus*와 *L. lactis*를 25℃, 30℃와 37℃에서 배양한 결과 배양 후 10시간, 25℃에서 최대의 균체를 생성하였다. 이에 반해 bulgaricin HJ와 lacticin HJ는 배양 후 10시간, 30℃에서 *Strep. agalactiae*에서 5120 AU/ml, *Staph. aureus*에서 320 AU/ml의 최대의 활성을 나타내었다. Bulgaricin HJ와 lacticin HJ는 *Staph. aureus* 보다 *Strep. agalactiae*에 대해 더욱 효과적임을 알 수 있었다.

*L. bulgaricus*와 *L. lactis*를 ammonium sulfate 침전, 투석, 동결건조를 통해서 조 bacteriocin을 조제하여 여러 가지 특성을 조사하였다. Bulgaricin HJ와 lacticin HJ는 여러 가지 단백질 분해효소에 의해 항균활성을 소실하였으므로 proteinous compounds임을 확인하였다. 또한 α-amylase, β-amylase, glucoamylase의 처리에도 활성을 소실하였으므로 두 bacteriocins이 bacteriocin의 분류 중 claas IV에 속함을 알 수 있었다. 100℃에서 pH에 따른 활성을 측정한 결과, 높은 pH value에서 급격히 활성을 소실하였으나 낮은 pH value에서는 100℃, 60분까지 활성이

남아 있음을 알 수 있었다. SDS-PAGE에 의해 두 bacteriocins가 대략 14kDa임이 확인되었으며 direct detection에 의해 bactericidal activity를 확인하였다.

Key words: Bacteriocin, mastitis pathogens, *Lactobacillus bulgaricus*, *Lactococcus lactis*

I. INTRODUCTION

Bovine mastitis can be defined as the inflammation of udder resulting from infection or trauma, which has been generally thought as a critical disease in cattle, hence resulting in reduced quantity and quality of milk and products from milk. These losses are primarily due to less milk production, increased veterinarian costs, increased cow mortality and discarded milk [5, 6, 12]. *Staphylococcus* and *Streptococcus* were the main two causative organism of mastitis. The causative organisms form 70 percent of mastitis pathogens. Currently, the bovine mastitis is one of major problems in dairy farming. The cows with bovine mastitis have been generally treated using antibiotics [7]. However, the widespread use of antibiotics, particularly for prophylactic application, is likely to be restricted in future. Also, antibiotics have been detected in the raw milk of cows with bovine mastitis by the antibiotics residue test. As a consequence, there is now a growing requirement for effective alternatives to treat mastitis.

The use of LAB and/or their metabolites for the related food is generally accepted by consumers as something natural and health-promoting [22]. That often produce a variety of antimicrobial compounds including lactic acid and acetic acids, diacetyl, hydrogen peroxide, carbon dioxide, alcohol, aldehyde and bacteriocins, all of which can antagonize the growth of spoilage bacteria and pathogenic bacteria. Among them, bacteriocins are biologically active peptides, proteins and protein complex produced by bacterial species acting against related species [8, 9]. Bacteriocins produced

by lactic acid bacteria (LAB) have been the subject of considerable research and industrial interest. In some instance, broad spectrum bacteriocins produced by lactic acid bacteria may provide valuable alternatives to antibiotics for the treatment. The proteinous nature of the bacteriocin contributes enzymatic degradation in the gastrointestinal tracts of human and animal [22]. From this reason, bacteriocins have been emerged as an alternative to the antibiotics to treat the cows infected with bovine mastitis pathogens. In this study, a LAB strains producing the bacteriocin killing mastitis pathogens were screened and the characteristics and the optimal production conditions of the bacteriocin were evaluated.

II. MATERIALS AND METHODS

1. Cell culture

Latobacillus bulgaricus and *Latococcus lactis* were cultured in MRS (Difco Lab., Detroit, U.S.A.) at 25, 30 and 37℃ for 24 h. Samples from the culture were taken at 1 h intervals for the measurement of cell mass and the activities of the produced substance. The cell mass was calibrated the wet basis on the values of optical density. Stock cultures were stored at -70℃ in MRS broth containing 33.3% glycerol for the next use.

The indicator strains, *Staphylococcus aureus* ATCC6538 and *Streptococcus agalactiae* ATCC14364, were incubated at 37℃. *Staph. aureus* ATCC6538 was grown in TSA (Trypticase Soy Agar) and *Strep. agalactiae* ATCC14364 was incubated in TSA with 5% defibrinated sheep blood.

2. Detection and activity assay of the antimicrobial substance

The substances produced by *L. bulgaricus* and *L. lactis* were detected by the agar well diffusion method and its activity was measured by a serial dilution method. For the agar well diffusion assay, 50 ㎕ of cell free supernatant broth was placed into the agar well inoculated with some gram-positive and gram negative bacteria, *Staph. aureus* ATCC6538 or *Strep. agalactiae* ATCC14364 as indicator strains [1]. The agar plate was left at 4℃ for 4 h and then incubated at 37℃ [4]. After 24 h, the agar plate was examined for zones of inhibition. The activities of substances were defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed as activity units (AU)

per milliliter. Assays were performed in triplicate.

3. Partial purification of the substance produced by *L. bulgaricus* and *L. lactis*

Partially purified substances were obtained using an ammonium sulfate precipitation method. The culture broth was centrifuged at 708 g at 4℃ for 30 min (Beckman Coulter, Inc., U.S.A.). The supernatant was adjusted to pH 6.5 to avoid isoelectric point, and then it was boiled for 10 min to remove the effect of proteases [3, 15]. The cell free supernatant was transferred into a beaker in an ice bath and 40% of ammonium sulfate was added. Subsequently, the mixture was centrifuged at 708 g at 4℃ for 45 min (Beckman Coulter, Inc., U.S.A.) and then the pellet was dissolved in 50 mM potassium phosphate buffer at pH 6.5. This solution was dialyzed using a membrane with a 3.5 kDa cut off (Spectrum Medical Inc., LA, U.S.A.) against the same buffer overnight. The dialyzed material (crude substance) was freeze-dried, then stored at -20℃ until use.

4. Characterization of the substance

To determine the antimicrobial activity spectrum, the substances produced by *L. bulgaricus* and *L. lactis* were tested with various gram-positive, gram negative bacteria and bovine mastitis pathogens.

For the determination of enzyme sensitivity, the crude substances produced by *L. bulgaricus* and *L. lactis* were treated with various enzymes with the concentration of 10 mg/ml. All enzymes (alcalase, proteinase K, aropase AP-10, glutaminase, protamax, peptidase R, neurase, flavourzyme,

glucoamylase, β-amylase, α-amylase, trypsin, catalase and pepsin) were dissolved in buffers as recommended by suppliers (Sigma, St. Louis, MO, U.S.A.). The samples of *L. bulgaricus* and *L. lactis* (180 ㎕) were mixed with 20 ㎕ portions of enzyme solutions (10 ㎎/㎖) and incubated at 37℃ for 2 h. The substances without enzyme was used as a negative control. After the incubation, the samples were adjusted pH 6-7, and heated for 5 min. The activities of treated samples were estimated by the agar well diffusion method. Also, dual effects with temperature and pH for the antimicrobial activity were determined. The pH was adjusted by the addition of either 5N HCl or 5N NaOH and then the samples were placed in a water bath at 100℃. Aliquot was taken after 15, 30, 45 and 60 min and the bacteriocin activities were measured by the serial dilution method [10, 21]. To determine the effect of metal ions on the antimicrobial substances, samples were treated with various metal ions such as $CoCl_2$, $ZnSO_4$, $MgSO_4$, $BaCl_2$, $CuSO_4$, $FeSO_4$, $CaSO_4$, and $MnSO_4$. Each metal ion was dissolved in deionized water and solution was added to the samples to obtain final concentration of 1 ㎎/㎖. Reaction mixture was incubated at 4℃ for 12 hours. The activity remaining in samples was also determined by the agar well diffusion method.

5. Determination of molecular weight of the antimicrobial substance by SDS-PAGE

The molecular size of the crude substances were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE

was carried out on a 15% acrylamide gel. For the estimation of the size, the molecular weight marker for SDS PAGE was used low molecular weight standard (Biorad).

Twenty $\mu\ell$ of sample was mixed with 5 $\mu\ell$ 5-fold-concent related sample buffer and boiled for 10 minutes. After electrophoresis at constant current of 20 mA for 3 h, the gel was removed and cut into two vertical parts. One part of the gel, containing sample and the molecular weight standard, was stained with Coomassie brilliant blue R250. The other, containing only the sample, was tested for antimicrobial activity using the method of Bhunie *et al.* [13, 16] with some modifications. The gel was fixed immediately by treating in 20% isopropanol and 10% acetic acid for 2 h and washed for 4 h in sterilized distilled water. The gel was then placed onto a petri dish and overlaid with 5 ml of 0.7% agar containing of the indicator strains (*Staph. aureus* ATCC6538 or *Strep. agalactiae* ATCC14364). The plate was incubated at 37℃ for 24 h and analyzed for the location of clear zone.

6. Mode of action against the mastitis pathogens

The substances were added into TSB broth culturing *Staph. aureus* ATCC6538 in the exponential growth phase and TSB broth containing 5% defibrinated sheep blood culturing *Strep. agalactiae* ATCC14364 in the exponential growth phase at 37℃. Samples were taken at every hour for the measurement of the absorbance at 660 nm. Colony forming units of *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364 by the treatment of the substance were counted.

III. RESULTS AND DISCUSSION

1. *L. bulgaricus*

1-1. Spectrum of antimicrobial activity

To determine the antimicrobial activity spectrum of the substance produced by *L. bulgaricus*, the cell free supernatant and partially purified substance were tested against various gram positive and gram-negative bacteria, such as other LAB and several pathogens (Table 1). The antimicrobial spectrum of the substance was broad and effective not only against LAB such as genus *Lactobacillus*, *Pediococcus* and *Leuconstoc* but also against gram-negative bacteria such as *Acetobacter* and *Pseudomonas*, especially, showing the inhibitory activity for mastitis pathogens such as *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364 [7]. Therefore, this substance was confirmed as a bacteriocin having antimicrobial activity. The substance produced by *L. bulgaricus* named as bulgaricin HJ.

Table 1. Antimicrobial spectrum of bulgaricin HJ.

Indicator strains	Activity[1] *L. bulgaricus*
G +	
Lactobacillus brevis	+++
Lactobacilus fermentum	+
Lactobacillus casei	+
Lactobacillus plantarum	+++
Lactobacillus helveticus CNRZ1096	+++
Lactobacillus delbrueckii	++
Lactococcus sp. JC3	-
Leuconostoc mensenteroides	+
Pediococcus acidilactis	+
Streptococcus mutans	+
Corynebacterium	++
Listeria monocytogenus	-
G -	
Acetobacter aceti	+++
E. coli DH5α	-
Pseudomonas synsantha	+++
Mastitis pathogens	
Streptococcus agalactiae ATCC14364	+++
Staphylococcus aureus ATCC6538	+++

1) The activity was expressed as the diameter of inhibition zone against each sensitive indicator. Degree of clarity of clear zone by growth inhibition: +, Inhibitory zone; -, no inhibition zone, +++: 1.0 cm > clear zone, ++: 0.5< clear zone< 0.9 cm +: 0.1< clear zone <0.5 cm.

1 2. Production of bulgaricus HJ

L. bulgaricus was cultured at the various culture temperatures of 25, 30 and 37℃ as shown in Fig. 1A. In case of *L. bulgaricus*, the cell growth at 30 and 37℃ were almost same, while the cell density at 25℃ was higher than those at 30 and 37℃. The maximum cell density reached was 1.1 g/l at 25℃. However, the cell growth patterns at 25, 30 and 37℃ were almost same. The logarithmic phase was observed after 4 hours of the growth and the stationary phase was observed after 10 hours of the culture. The *L. bulgaricus* started to produce the bacteriocin from 6 h of the cultivation. Figs. 1B and C show the activity of the bulgaricin HJ during the cultivation of *L. bulgaricus* at different temperatures. The maximum activity of the bulgaricin HJ with the indicator *Strep. agalactiae* ATCC14364 reached 5120 AU/ml for 5 hours (from 10 h to 15 h) in the culture at 30℃ (Fig. 1B). The activities of bulgaricin HJ reached 2560 AU/ml for two hours (from 11 h to 13 h) in the culture at 37℃, and about 160 AU/ml for three hours (from 10 h to 13 h) in the culture at 25℃. The highest bacteriocin activities were showed at the stationary phase of the *L. bulgaricus* cultures, which results were similar to previous reports [2, 16]. Therefore, the optimal condition for the production of the bulgaricin HJ was determined at 30℃ and in the early stationary phase from 10 h to 15 h. Fig. 1C shows the activity of the bacteriocin obtained from various culture temperatures with *Staph. aureus* ATCC6538 as the indicator. The maximum activity of the bacteriocin reached 320 AU/ml during the culture time from 10 h to 15 h at 30℃ and 160 AU/ml during the culture time from 8 h to 18 h at 25℃ and 160 AU/ml during the culture time from 4 h

to 16 h at 37℃. The optimal condition for the production of the bacteriocin with *Staph. aureus* ATCC6538 was exactly same to those with *Strep. agalactiae* ATCC14364.

The bulgaricin HJ was more effective to *Strep. agalactiae* ATCC14364 rather than *Staph. aureus* ATCC6538 as shown in Fig. 1. The maximum activity of the bulgaricin HJ was obtained at 30℃ and the culture of *L. bulgaricus* in early stationary phase as a typical secondary metabolite production [15, 20]. After late stationary phase of the culture, rapid decreases in the activities of the bacteriocin was observed due to the degradation of the bacteriocin by extra cellular proteolysis enzymes showing same trends of previous report [18].

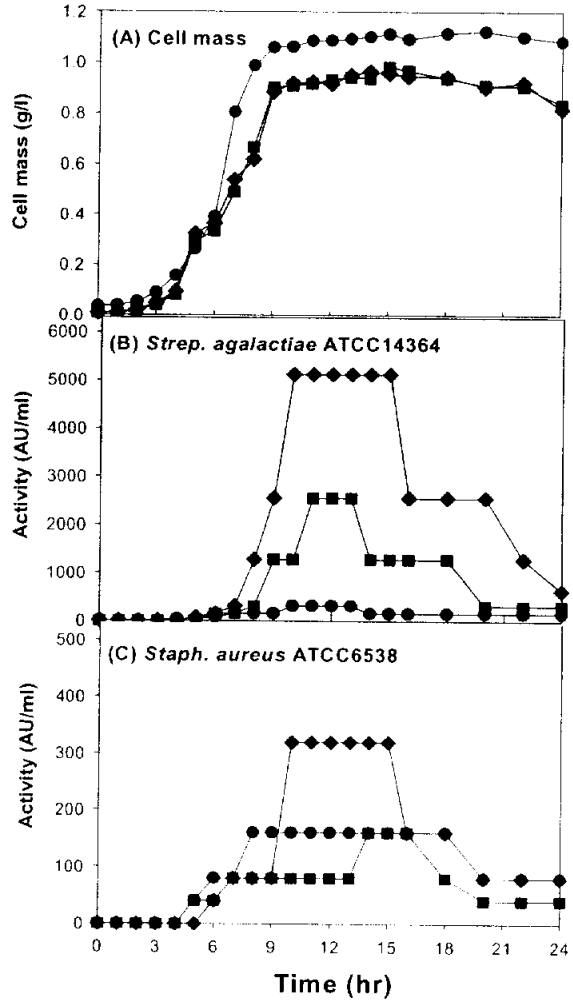

Fig. 1. Time course on the cell mass and the production of the bacteriocin of *L. bulgaricus against Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538. The cell density (A) and the change of bacteriocin activity were monitored every hour against *Strep. agalactiae* ATCC14364 (B) and *Staph. aureus* ATCC6538 (C). Symbols: ●, 25℃ ◆, 30℃ ■, 37℃.

1 3. Characterization of bulgaricin HJ

1 3 1. The activity of the bulgaricin HJ exposed with enzymes

The activities of bulgaricin HJ in the buffer containing the enzymes were measured as shown in Table 2. Proteolysis enzymes (aropase AP-10, protamax, proteinase K, neurase, alcalse and pepsin) caused the complete loss of the bacteriocin activity. This indicates that the bateriocin was the proteinous substances. In addition, when bulgaricin HJ was treated with α-amylase, β-amylase and glucoamylse, bulgaricin HJ activity against the indicator of *Staph. aureus* ATCC6538 was lost. Also, when bulgaricin HJ was treated with β-amylase, the bulgaricin HJ activity against *Strep. agalactiae* ATCC14364 was lost. The loss of an antimicrobial activity of bulgaricin HJ by α-amylase, β-amylase and glucoamylse indicates that carbohydrate composition on the bulgaricin HJ is related to the antimicrobial activity of the bacteriocin. Therefore, the bacteriocin was considered to be a class IV bacteriocin according to reports [1, 13, 19].

1 3 2. The activity of the bulgaricin HJ exposed with heat treatment

The activity of the bulgaricin HJ was lost at the high pH with heat treatment at 100℃ as shown in Fig. 2. The activity of 100% was referred to the activity at the pH 7 and 30℃ as shown in Fig. 1B and C. Hundred % of the bacteriocin activity was maintained at pH 2-7 with heat treatment at 100℃ for 30 min. However, the activity decreased to 50% after 60 min of heat treatment. In the alkali condition (pH 10-11), the decrease of the activity was observed with heat treatment for short time. This result indicates that bulgaricin HJ was stable in acidic and nature

conditions (pH 2-7) with heat treatment for 30 min [13, 17].

1-3-3. Sensitivity of bulgaricin HJ to metal ions

As shown Table 3, bulgaricin HJ activity was not affected by the treatment of various metal ions. This result indicates that common bivalent ions was not related bulgaricin HJ activity.

1-4. Determination of molecular weight of bulgaricin HJ by SDS-PAGE

The apparent molecular weight of bulgaricin HJ was determined by SDS-PAGE as shown in Fig. 3. Lane A shows a standard marker and lane B, C and D show bulgaricin HJ samples. Lane A and lane B were stained by Coomassie brilliant blue. Lane B shows the molecular weight of bulgaricin HJ samples was approximately 14 kDa and the clear bands by the bacteriocin were observed on the lane C and lane D. Lane C and D were overlaid with indicator strains, *Strep. agalactiae* ATCC14364 (Lane C) and *Staph. aureus* ATCC6538 (Lane D). Lane C and D also show that the molecular weight of the bacteriocin from *L. bulgaricus* was about 14 kDa.

1-5. Mode of action against mastitis pathogens

The colony forming units (CFU) of *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538 with bulgaricin HJ were measured (Fig. 4). Five hours of incubation with indicator strains and bulgaricin HJ resulted in the decrease of live cell number. When *Strep. agalactiae* ATCC14364 was treated with bulgaricin HJ, the cell number decreased from $1.35X10^{13}$

CFU/ml to $3.8X10^6$ CFU/ml. Similarly, when *Staph. aureus* ATCC6538 was treated with bulgaricin HJ, the cell number decreased from $2X10^{13}$ CFU/ml to $9.2X10^7$ CFU/ml. However, the optical density increased for 2 h then kept constant in both case of indicator strains as shown in Fig. 4. These results indicate bulgaricin HJ acted in bactericidal mode [11, 17].

Table 2. The sensitivity of bulgaricin HJ to the various enzymes.

Enzymes	*L. bulgaricus*	
	Staph. aureus ATCC6538	*Strep. agalactiae* ATCC14364
Alcalase	-	
Aropase AP-10	-	-
Catalase	+	+
Flavourzyme	-	+
Glutaminase	-	+
Neurase	-	-
Pepsin	-	-
Protamax	-	
Proteinase K		-
Trypsin		+
α-amylase		+
β-amylase	-	
Glucoamylase	-	+

Staph. aureus ATCC6538 and *Strep. agalactiae* ATCC14364 were used as indicators. +, Detection of inhibitory zone observed; -, no inhibition zone.

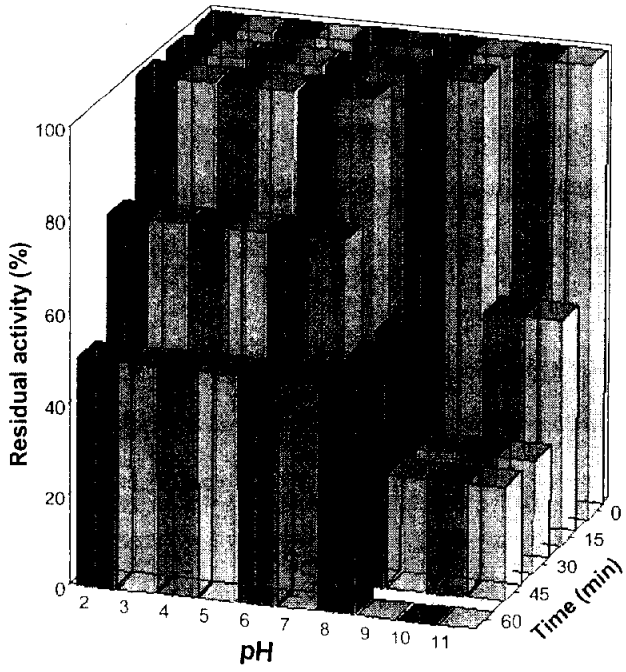

Fig. 2. Effects of pH and heat treatment at 100℃ on the bactericidal activity of the bacteriocin produced by *L. bulgaricus* against *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538.

Table 3. Effect of metal ions on stability of bulgaricin HJ

Metal ions	Residual activity (%)
$BaCl_2$	100
$CaSO_4$	100
$CoCl_2$	100
$CuSO_4$	100
$FeSO_4$	100
$MgSO_4$	100
$MnSO_4$	100
$ZnSO_4$	100

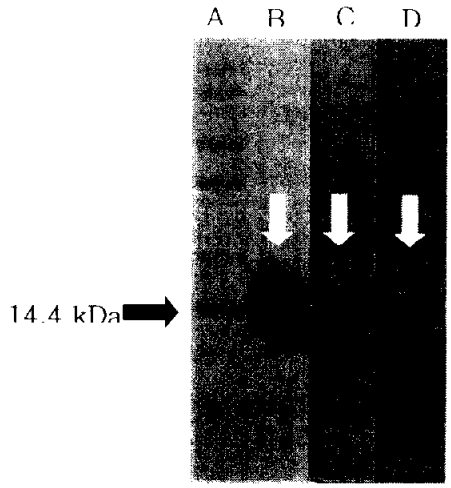

Fig. 3. Polyacrylamide gel (15%) electrophoresis of bulgaricin HJ.
Lane A: standard marker with 97.4, 66.2, 45, 31, 21.5, 14.4 kDa (Biorad, low molecular weight), Lane B: bulgaricin HJ sample stained by Coomassie brilliant blue R250. Lane C and D: bulgaricin HJ sample overlaid with indicator strains, *Strep. agalactiae* ATCC14364 (lane C) and with indicator strain *Staph. aureus* ATCC6538 (lane D). The arrow indicates the location of bulgaricin HJ.

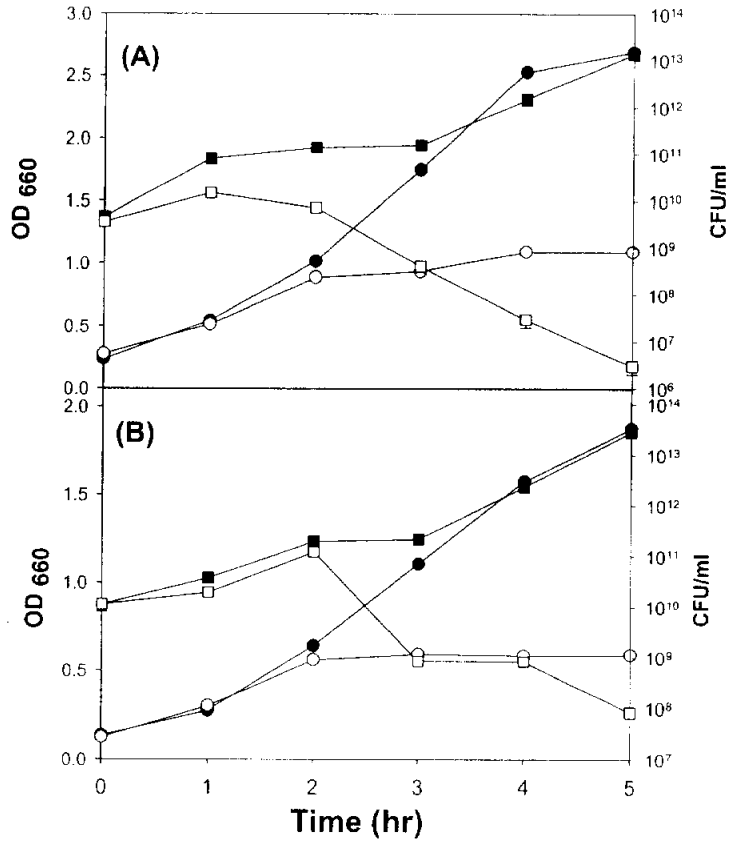

Fig. 4. The activities CFU of the bacteriocin produced by *L. bulgaricus* against indicator strains *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538. A) The inhibitory effect of bulgaricin HJ against *Strep. agalactiae* ATCC14364; B) The inhibitory effect of the bacteriocin against *Staph. aureus* ATCC6538. Symbols: ●, Optical density of control; ○, Optical density of culture treated by bulgaricin HJ; ■, CFU of control; □, CFU of culture treated by bulgaricin HJ.

2. *L. lactis*

2 1. Spectrum of antimicrobial activity

To determine the antimicrobial activity spectrum of the substance produced by *L. lactis*, the cell free supernatant and partially purified substance were tested against various gram-positive and gram-negative bacteria, such as other LAB and several pathogens (Table 4). The antimicrobial spectrum of the substance was broad and effective not only against LAB including genus *Lactobacillus* but also against gram-negative bacteria such as *Pseudomonas*, especially, showing the inhibitory activity for mastitis pathogens such as *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364 [7]. Therefore, this substance was confirmed as a bacteriocin having antimicrobial activity. The substance produced by *L. lactis* named as lacticin HJ. The bulgaricin HJ was more effective against *Lactobacillus brevis*, *Acetobacter* and, *Corynebactrium* than the lacticin HJ. Lacticin HJ was more effective against *Latobacillus fermentum* than bulgaricin HJ.

Table 4. Antimicrobial spectrum of lacticin HJ

Indicator strains	Activity[1] *L. lactis*
G +	
Lactobacillus brevis	++
Lactobacilus fermentum	++
Lactobacillus casei	+
Lactobacillus plantarum	+++
Lactobacillus helveticus CNRZ1096	+++
Lactobacillus delbrueckii	++
Lactococcus sp. JC3	
Leuconostoc mensenteroides	+
Pediococcus acidilactis	+
Streptococcus mutans	+
Corynebacterium	+
Listeria monocytogenus	-
G	
Acetobacter aceti	+
E. coli DH5α	
Pseudomonas synsantha	+++
Mastitis pathogens	
*Streptococcus agalactiae*ATCC14364	+++
Staphylococcus aureus ATCC6538	+++

1) The activity was expressed as the diameter of inhibition zone against each sensitive indicator. Degree of clarity of clear zone by growth inhibition: +, Inhibitory zone; , no inhibition zone, +++: 1.0 cm > clear zone, ++: 0.5< clear zone< 0.9 cm +: 0.1< clear zone <0.5 cm.

2 2. Production of lacticin HJ

L. lactis was cultured at the various culture temperatures of 25, 30 and 37℃. *L. lactis*, the cell growth and activity of the lacticin HJ were shown as Fig. 5. The logarithmic phase and the production of lacticin HJ observed after 4 hours. The cell mass pattern was same as *L. bulgaricus*. The maximum cell mass reached at 1.1 g/ℓ at 25℃. The maximum activity of lacticin HJ against *Strep. agalactiae* ATCC14364 reached at 5120 AU/ml during the cultivation from 9 h to 16 h at 30℃ and against *Staph. aureus* ATCC6538 reached at 320 AU/ml during the cultivation from 7 h to 20 h at 30℃. The maximum activities of the lacticin HJ against *Strep. aglactiae* ATCC14364 for 7 hours and against *Staph. aureus* ATCC6538 at 30℃ were maintained for 13 hours.

The lacticin HJ was more effective to *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538 rather than bulgaricin HJ. The maximum activity of lacticin HJ was about two times longer than the maximum activity of bulgaricin HJ against *Staph. aureus* ATCC6538. Also, lacticin HJ activity was observed for 8 hours, whereas the bulgaricin HJ was observed for 6 hours.

Two bacteriocins were more effective to *Strep. agalactiae* ATCC14364 rather than *Staph. aureus* ATCC6538 as shown in Figs. 1 and 5. The maximum activity of the two bacteriocins were obtained at 30℃ and the culture of *L. bulgaricus* and *L. lactis* in early stationary phase as a typical secondary metabolite production [15, 20]. After the late stationary phase of the culture, rapid decreases in the activity of the lacticin HJ was observed due to the degradation of the lacticin HJ by extra cellular proteolysis

enzymes showing same trends of *L. bulgaricus* [18].

2 3. Characterization of lacticin HJ

2-3 1. The activity of lacticin HJ exposed with various enzymes

The effect of various enzymes on lacticin HJ was shown Table 5. The activity of lacticin HJ was complete loss by various proteolysis enzymes (alcalse, aropase AP-10, flavourzyme, neurase, pepsin, protamax and, proteinase K). This indicates that the bateriocin was the proteinous substances. The lacticin HJ activity was compete lost by glucoamylase against *Staph. aureus* ATCC6538 and *Strep. agalactiae* ATCC14364.

In addition, when lacticin HJ was treated with β-amylase and glucoamylase, lacticin HJ activity against the indicator of *Strep. agalactiae* ATCC14364 was lost. The lacticin HJ activity was not affected by the treatment catalase, α-amylase. Also, the activity of lacticin HJ against *Staph. aureus* ATCC6538 was remained treatment β-amylase, and against *Strep. agalactiae* ATCC14364 was not affected trypsin. The loss of an antimicrobial activity of the bacteriocin by β-amylase and glucoamylse indicates that carbohydrate composition on the bacteriocin is related to the antimicrobial activity of the bacteriocin. Therefore, lacticin HJ was considered to be a class IV bacteriocin according to reports [1, 13, 19].

2 3 2. The activity of the lacticin HJ exposed with heat treatment

Lacticin HJ exhibited consistent stability over the wide range of pH (from pH 2 to pH 11) for 15 min at 100℃ (Fig. 7). The activity of 100% was referred to the activity at the pH 7 and 30℃ as shown in Fig. 5B

and C. The activity of lacticin HJ was remained for 30 min at 100℃ against *Strep. agalactiae* ATCC14364, whereas lacticin HJ activity was remained for 15 min at 100℃ against *Staph. aureus* ATCC6538. In acidic conditions, the activity of lacticin HJ was retained for 60 min at 100℃ against indicator strains. However, the loss of lacticin HJ activity was observed after 60 min at 100℃ against indicator strains. The lacticin HJ was more stable in acidic conditions (pH 2-8) than that in alkalic conditions (pH 9-11). The activity of lacticin HJ against *Strep. agalactiae* ATCC14364 was more than that against *Staph. aureus* ATCC6538 in acidic conditions at 100℃. This result indicates that lacticin HJ was stable in acidic and nature conditions (pH 2-7) with heat treatment for 30 min [13, 17].

2 3 3. Sensitive of lacticin HJ to metal ions

To test the effect of common bivalent ions on lacticin HJ activity, lacticin HJ mixed with metal ions solutions. However, metal ions did not affect lacticin HJ activity (Table 6).

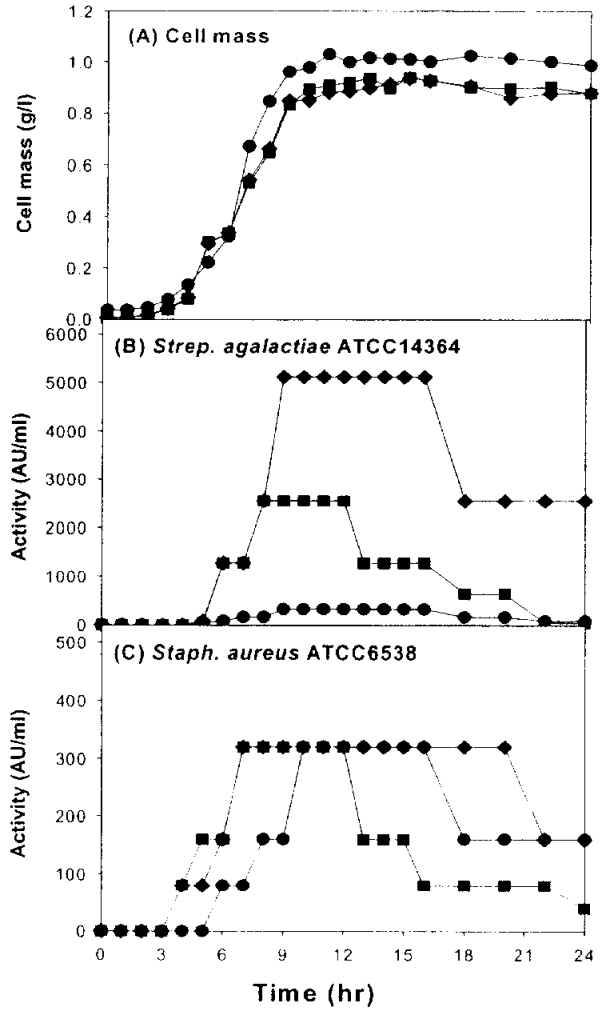

Fig. 5. Time course on the cell mass and the production of the bacteriocin of *L. lactis* against *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538. The cell density (A) and the change of bacteriocin activity were monitored every hour against *Strep. agalactiae* ATCC14364 (B) and *Staph. aureus* ATCC6538 (C). Symbols: ●, 25℃ ◆, 30℃ ■, 37℃.

Table 5. The sensitivity of lacticin HJ to the various enzymes.

Enzymes	*L. lactis*	
	Staph. aureus ATCC6538	*Strep. agalactiae* ATCC14364
Alcalase	-	-
Aropase AP-10	-	
Catalase	+	+
Flavourzyme	-	
Glutaminase	-	+
Neurase		
Pepsin	-	
Protamax	-	-
Proteinase K		-
Trypsin		+
α-amylase	+	+
β-amylase	+	
Glucoamylase		

Staph. aureus ATCC6538 and *Strep. agalactiae* ATCC14364 were used as indicators. +, Detection of inhibitory zone observed; -, no inhibition zone.

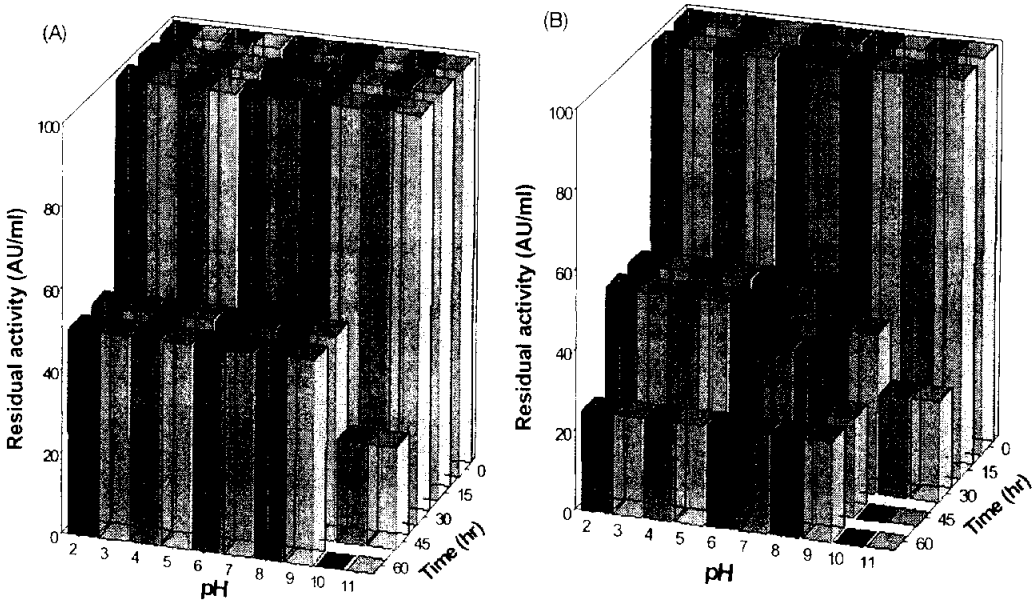

Fig. 6. Effects of pH and heat treatment at 100℃ on the bactericidal activity of the bacteriocin produced by *L. lactis* against *Strep. agalactiae* ATCC14364 (A) and *Staph. aureus* ATCC6538 (B).

Table 6. Effect of metal ions on stability of lacticin HJ

Metal ions	Residual activity (%)
$BaCl_2$	100
$CaSO_4$	100
$CoCl_2$	100
$CuSO_4$	100
$FeSO_4$	100
$MgSO_4$	100
$MnSO_4$	100
$ZnSO_4$	100

2-4. Determination of molecular weight of lacticin HJ by SDS-PAGE

The apparent molecular weight of lacticin HJ was determined by SDS-PAGE as shown in Fig. 7. Lane A shows a standard marker and lane B, C and D show lacticin HJ samples. Lane A and lane B were stained by Coomassie brilliant blue. Lane B shows the molecular weight of lacticin HJ samples was approximately 14 kDa and the clear bands by the lacticin HJ were observed on the lane C and lane D. Lane C and D were overlaid with indicator strains, *Strep. agalactiae* ATCC14364 (Lane C) and *Staph. aureus* ATCC6538 (Lane D). Lane C and D also show that the molecular weight of the bacteriocin from *L. lactis* was 14 kDa.

2-5. Mode of action against mastitis pathogens

The colony forming units (CFU) of *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538 with lacticin HJ were measured (Fig. 8). Five hours of incubation with indicator strains and the lacticin HJ resulted in the decrease of live cell number.

When *Strep. agalactiae* ATCC14364 was treated with lacticin HJ, the cell number decreased from $1.52X10^{13}$ CFU/ml to $1.8X10^{6}$ CFU/ml. Similarly, when *Staph. aureus* ATCC6538 was treated with lacticin HJ, the cell number decreased from $3X10^{13}$ CFU/ml to $1.35X10^{5}$ CFU/ml. However, the optical density increased for 2 h then kept constant in both case of indicator strains as shown in Fig. 8. These results indicate that lacticin HJ acted in bactericidal mode [11, 17].

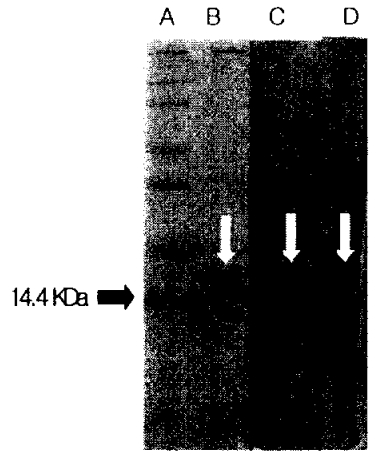

Fig. 7. Polyacrylamide gel (15%) electrophoresis of the lacticin HJ.
Lane A: standard marker with 97.4, 66.2, 45, 31, 21.5, 14.4 kDa (Biorad, low molecular weight), Lane B: lacticin HJ sample stained by Coomassie brilliant blue R250. Lane C and D: lacticin HJ sample overlaid with indicator strains, *Strep. agalactiae* ATCC14364 (lane C) and with indicator strain *Staph. aureus* ATCC6538 (lane D). The arrow indicates the location of lacticin HJ.

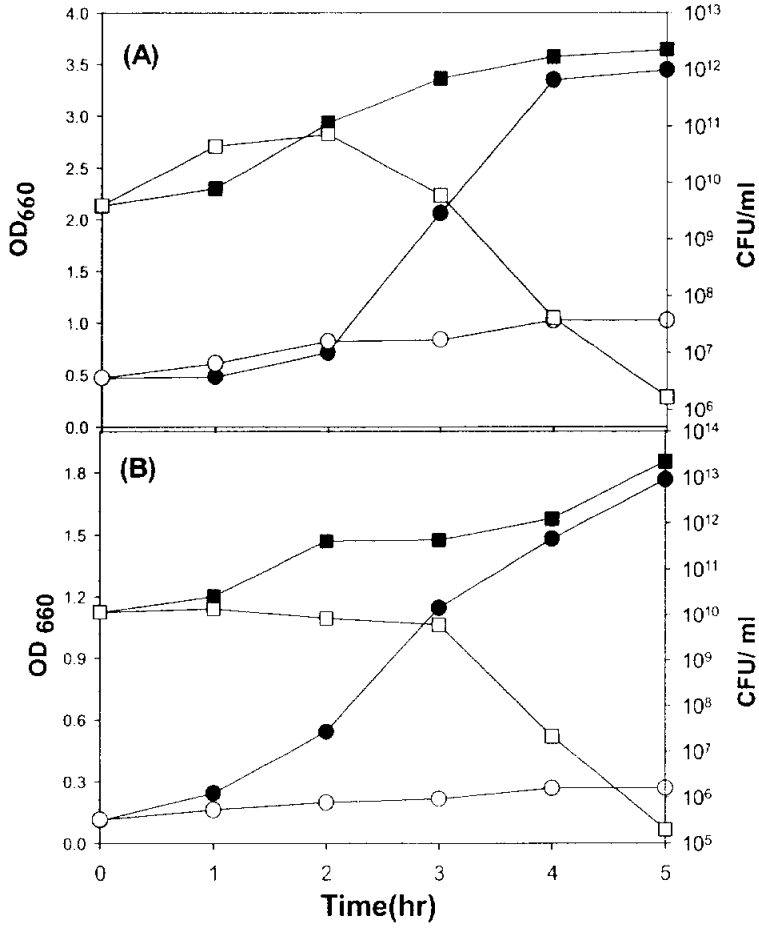

Fig. 8. The activities CFU of the bacteriocin produced by *L. lactis* against indicator strains *Strep. agalactiae* ATCC14364 and *Staph. aureus* ATCC6538. A) The inhibitory effect of the lacticin HJ against *Strep. agalactiae* ATCC14364; B) The inhibitory effect of the lacticin HJ against *Staph. aureus* ATCC6538. Symbols: ●, Optical density of control; ○, Optical density of culture treated by lacticin HJ; ■, CFU of control; □, CFU of culture treated by lacticin HJ.

IV. CONCLUSIONS

The antimicrobial substances produced by *Lactobacillus bulgaricus* and *Lactococcus lactis* were inactivated by protease and showed the inhibitory activity against *Staphylococcus aureus* ATCC6538, *Streptococcus agalactiae* ATCC14364, some gram-positive and gram-negative bacteria. The substance showed characteristics of as a bacteriocins. Therefore, the substance produced by *L. bulgaricus* named as bulgaricin HJ and the substance produced by *L. lactis* named as lacticin HJ. The optimal condition for the production of the bulgaricin HJ was determined at 30℃ and in the early stationary phase from 10 h to 15 h. The maximum activity of lacticin HJ against *Strep. agalactiae* ATCC14364 reached during the cultivation from 9 h to 16 h at 30℃ and against *Staph. aureus* ATCC6538 reached during the cultivation from 7 h to 20 h at 30℃.

Two bacteriocins production started in the exponential phase and reached a maximum at the early stationary phase in the culture of *L. bulgaricus* and *L. lactis*. The antimicrobial activities of bulgaricin HJ and lacticin HJ were stable in acidic and nature conditions (pH 2-7) even with heat treatment at 100℃, whereas, it was lost at high pH values (pH 10-11) combined the heat treatment at 100℃. The mode of action for bulgaricin HJ and lacticin HJ were bactericidal and the molecular weight determined by SDS-PAGE and overlay method were about 14 kDa.

V. ACKNOWLEDGEMENT

먼저 부족한 저에게 석사 생활을 잘 마무리 할 수 있도록 지도와 격려를 아끼지 않으신 김성구 교수님께 머리숙여 감사드립니다. 또한 많은 가르침을 전해주신 공재열 교수님, 이형호 교수님, 공인수 교수님, 박남규 교수님과 본 논문이 완성되도록 많은 지도를 해주신 김중균 교수님과 홍용기 교수님께도 감사의 마음을 전합니다. 또한 늘 관심을 가지고 격려해주신 동의대 남수완 교수님과 실험실 가족들에게도 감사드립니다.

실험실 생활에서 늘 힘이 되어준 김미령 박사님, 서재관 박사님, 손정화 박사님, 김영화, 조경진, 정일형, 유선희, 김정화 선배님께도 고마운 마음을 전합니다. 큰 사랑을 주신 서효진, 임성미 선배님의 앞날이 행복하기를 기원합니다. 또한 열심히 공부하고 있을 배선명 선배님도 좋은 결실이 있기를 바랍니다. 항상 나의 곁에서 말없이 힘이 되어준 김지현에게도 고마움의 마음을 전합니다.

든든한 후배들인 박소진, 김소영, 최은민, 김윤임, 하유미, 남창우 그리고 Wu Jian-Rong 선배님도 좋은 결실이 있기를 바랍니다. 또한 따뜻한 격려를 해주신 생물공학과 선후배님들께도 진심으로 감사드립니다. 언제나 나를 믿어준 동기 윤정, 은미 그 외의 98 동기들에게도 고마움을 전합니다.

힘들 때 웃을 수 있도록 많은 격려해준 소중한 친구 옥순이의 앞날에 무한한 발전이 있기를 바랍니다. 이제껏 친딸처럼 걱정해주신 강현근 선생님에게 도 감사의 마음을 전합니다.

공부하는 언니를 항상 걱정해준 동생 은진이와 한결같이 누나를 믿어준 동생 재홍이에게도 고마움과 사랑의 마음을 전합니다. 부족한 큰 딸을 묵묵히 믿어주시며 고생을 마다하시지 않으신 사랑하는 아버지와 어머니께 감사의 마음과 고마움을 담아 이 논문을 바칩니다.

마지막으로 언제나 나를 믿어주며 옆에서 지켜준 나의 선배야, 병진 선배에게도 고마움과 사랑의 마음을 전합니다.

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