Characterization of action mechanism of glycinecin A, a bacteriocin derived from *Xanthomonas campestris* pv. *glycines 8ra* on phytopathogenic *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

The ability of glycinecin A, a bacteriocin derived from *Xanthomonas campestris* pv. *glycines 8ra*, to kill closely related bacteria has been demonstrated previously by Heu (Heu *et al.* 2001). In the present study, we aimed at determining the glycinecin A induced cause of death of sensitive cells. Treatment with glycinecin A caused slow dissipation of membrane potential in comparison to valinomycin, a cell membrane potential disruptor. In contrast glycinecin A induced the depletion of the pH gradient (ΔpH) of *Xanthomonas* cells as rapidly as did nigericin, a H^+/K^+ exchanger, that rapidly reversed the pH of the cells to that of the medium. Meanwhile glycinecin A did not induce the depletion of ΔpH of *E. coli* cells, which indicate that glycinecin A may need a specific receptor for its binding and action. Glycinecin A treatment also induced leakage of potassium ions, from *X. campestris* pv. *vesicatoria* YK93-4 cells, but not induced leakage of other ions such as Mg^{2+} and PO_4^{3-}. The induction of potassium leakage due to glycinecin A is faster and higher than the induction caused by valinomycin. The bacteriocin killed sensitive bacterial cells in a dose-dependent manner, sensitive cells were killed within two hours of incubation, most strictly due to the potassium ion efflux caused by glycinecin A. These results suggest that the bactericidal mechanism of action of glycinecin A is correlated with the permeability of membranes to hydroxyl and potassium ions, leading to the lethal activity of the bacteriocin on the target bacteria.

Key words: Bacteriocin, *Xanthomonas*, Glycinecin A

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Ⅰ. INTRODUCTION

Bacteriocins, extracellular proteinaceous bactericidal substances that are produced by many species of bacteria, trigger the killing of strains or species closely related to their producers (Tagg *et al*., 1976; Klaenhammer *et al.*, 1988).

The historical definition of bacteriocins is applied to the most well known group, the colicins, which are produced principally by the gut inhabitant *Escherichia coli*. Originally, the colicins, antibiotic-like compounds were discovered first as bacteriocins by Gratia in 1925. Their narrow specificity of action and their proteinaceous nature distinguish them from other antibiotics (Daw *et al.* 1996).

The names of bacteriocins are generally derived from the producing genus or species. One prevalent naming is to use the foot of the species plus "-cin", following by strain designation, for example syringacin 4-A, which conveys the information that *Pseudomonas ryringae* pv. *syringae* 4-A is the producer (Smidt *et al.,* 1982)

Bacteriocins have been found in all major lineages of bacteria, and more recently, have been described as universally produced by some members of *Archaea* (Torreblanca *et al.,* 1989). According to Klaenhammer, 99% of all bacteria produce at least one bacteriocin and the reason we have not isolated more is that few researchers have looked for them (Klaenhammer *et al.,* 1988). In phytophathogenic bacteria, many bacteriocins have been discovered. Among plant pathogenic *Pseudomonads*, about 10 species or subspecies have been reported to produce bacteriocins (Lavermicocca *et al.* 2002), and bacteriocins have also been discovered in other plant pathogens such as: *Agrobacterium radiobacter* (Robert *et al.* 1977), *Corynebacterium michiganense (*Echandi, 1976*), Erwinia carotovora* (Crowley *et al*. 1980*), E. chrysanthemi* (Echandi *et al.,* 1979), *Pseudomonas syringae* (Smidt *et al.,* 1982*), Ralstonia solanacearum* (Chen, 1984) *Xanthomonas oryzae* (Mew *et al*. 1982), *Xanthomonas campestris pv. glycines* (Fett *et al*., 1987). These bacteriocins have been used in different ways for bio-controls of plant diseases (Lavermicocca *et al.* 2002).

Bacteriocins have been broadly used for food preservation (Garneau *et al.,* 2002),

identification of bacteria and biological controls (Kerr *et al.,* 1974). There is a tremendous interest in their use as a novel means to ensure the safety of foods. Biochemical properties of nisin, a bacteriocin produced by *Lactococcus lactis,* have been well studied, and nisin is known as a safe bio-preservative without any adverse effects on human health. Nisin has been employed for over 50 years and is currently approved in more than 60 countries for use as an adjunct to thermal processing to prevent spoilage by thermophilic spore formers (Montvelle *et al.,* 1995). Recently bacteriocins have also been used for veterinary medicine, and in dentistry (Garneau *et al*., 2002).

The modes of action of bacteriocins vary (Braun *et al.,* 1994; Montvelle *et al.,* 1995). Some bacteriocins inhibit the synthesis of macromolecules, such as colicin E3, E4 and E6, which specifically inhibit protein synthesis with RNase activities (Olatomirin *et al.,* 2002; Zaeivach *et al.,* 2002). Others have nuclease activity: colicin E2, E7, E8, E9 and pyocin S3 induce DNA degradation (Kolade *et al*., 2002; Duport *et al.*, 1995; Ringose, 1970), and colicin E5 cleaves a specific group of tRNAs (Masaki *et al.,* 1997). Meanwhile, enterocin EJ97 has a bacteriolytic effect on *E. faecalis* S-47 (Galvez *et al*., 1998).

The cytoplasmic membrane of sensitive cells is the biological target of bacteriocins. The changes in membrane permeability induced by bacteriocins decrease or deplete the proton motive force (PMF). PMF is an electrochemical gradient composed of a membrane potential ($\Delta\Psi$) and a pH gradient (ΔpH). These gradients serve as the driving force for many vital energy dependent cellular processes (Montville *et al.,* 1995). Two-component bacteriocins, such as lacticin 3147, plantaricin EF, plantaricin JK, and lactococcin G, require the complementary actions of two components for activity, and target the cell membrane (McAuliffe O. *et al.,* 1998; Moll G. *et al.,* 1996 and 1999). Lactococcin G selectively forms potassium channels in target bacterial membranes (Moll *et al.,* 1996), and the plantaricins EF and JK form pores in the membranes of target cells, dissipating the transmembrane electrical potential and pH gradient (Moll *et al*., 1999). The cytoplasmic membrane is the primary target of colicins A, E1, K, Ia, and Ib (Daw *et al*., 1996; Duche, 2002; Hechard *et al*., 2002). These and other related colicins disrupt transport and induce the leakage of ions, such as potassium and magnesium ions, by

forming voltage-dependent channels in phospholipid bilayers, destroying the potential of the cell. These actions result in the inhibition of protein or nucleic acid biosynthesis and uncoupled electron transport from active transport of thiomethyl-β-D-galactoside and potassium. The loss of these ions has been implicated as the primary cause of cell death (Daw *et al*., 1996). The bactericidal activities of enterocoliticin and serracin P have been shown to have a phage tail-like action that forms pores in target cell membranes (Jabrane *et al.*, 2002; Strauch *et al.*, 2001).

Producer strains prevent the pore formation or enzymatic activities of the acteriocins by producing immunity proteins. The mechanisms may not be well identified yet. However, this may be accomplished by shielding of the receptor protein, by competitive interaction with the bacteriocin molecules, by binding the active sites of bacteriocins, or by blocking the pores (Kolade *et al*., 2002; Abee, 1995)

Xanthomonas campestris pv. *glycines* 8ra has antibacterial activity against most phytopathogenic *Xanthomonas* species tested, including *X. axonopodis*, *X. campestris* pv. *campestris*, *X. campestris* pv. *citri*, *X. campestris* pv. *pruni*, and *X. campestris* pv. *vesicatoria* (Woo *et al.*, 1998). *X. campestris* pv. *glycines* 8ra produces a bacteriocin named glycinecin A, a heterodimeric protein consisting of 39-kDa and 14-kDa subunits encoded by the *glyA* and *glyB* genes, respectively. The coexpression of the two subunits in the same host is essential for the activity of this bacteriocin (Heu *et al.,* 2001). Glycinecin A treatment was as effective in the control of *X. oryzae* pv. *oryzae* and *X. campestris pv. vesicatoria* as chemical treatment with copper hydroxide (Jeon *et al*., 2001). A chimeric glycinecin A, consisting of concatemerized glyA and glyB, has been demonstrated to have bactericidal activity comparable to that of wild-type glycinecin A, and the protein was more stable with varying pH and temperature than the wild-type protein (Kim *et al.,* 2001). Understanding the mode of action of bacteriocin will be important to allow effective utilization of this protein.

The purpose of this study was to determine the mode of action of glycinecin A on *Xanthomonas campestris* pv. *vesicatoria* cells. Purified chimeric glycinecin A was used in this study.

II. MATERIALS AND METHODS

1. Chemicals, glycinecin A, bacterial strains, and growth conditions

LB broth, nutrient agar (NA), potassium N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (K-HEPES), 3,3-dipropylthiocarbocyanine [$DiSC_3(5)$]; 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxylfluorescein (BCECF), and the K ionophores valinomycin and nigericin (Nig) were purchased from Sigma (St. Louis, MO).

Chimeric glycinecin A (Scheme 1) was expressed and purified from *E. coli* DH5α transformed with the *glyA/B* gene as described previously (Heu *et al.*, 2001). *Xanthomonas campestris* pv. *vesicatoria* YK93-4 was used as a sensitive organism to test the bactericidal mode of action. *E. coli* cultures were grown in LB broth at 37ºC and *Xanthomonas campestris* pv. *vesicatoria* was grown in nutrient both (NB) at 28ºC.

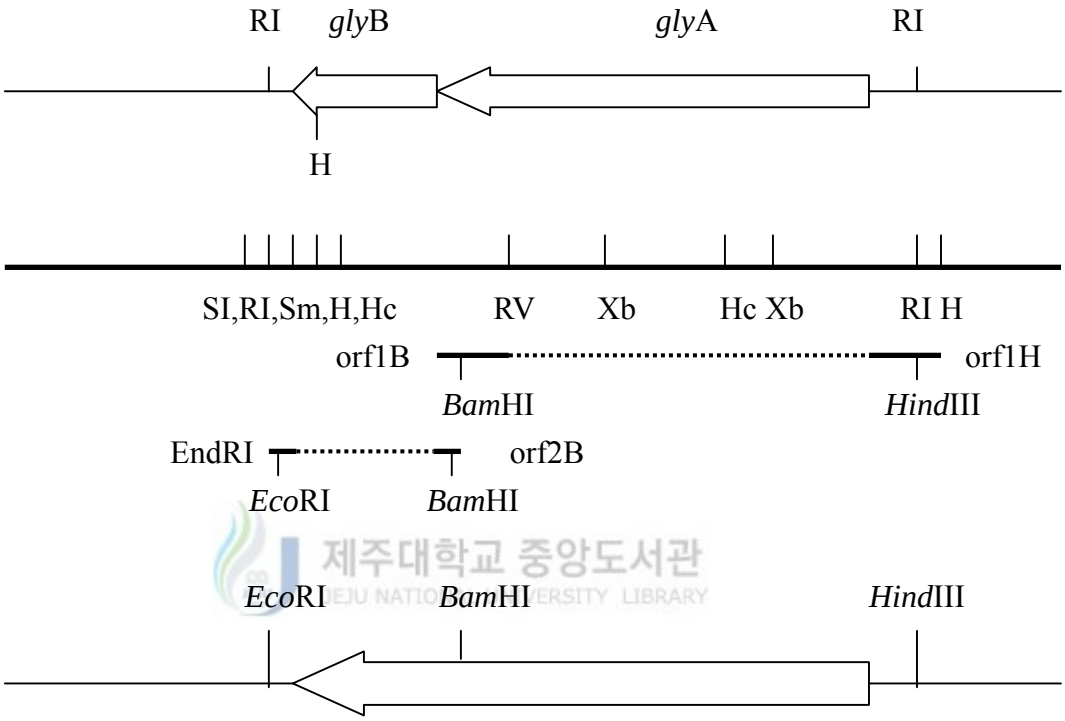
2. Purification of glycinecin A

Glycinecin A was expressed in *E. coli* DH5α transformed with pSGEB and purified (Scheme 2) as described previously (Heu *et al.*, 2001) with slight modifications. Briefly, *E. coli* DH5α carrying the *glyA/B* gene (pSGEB) was grown for 48 h at 37ºC in LB containing ampicillin (50 μg/ml). The bacterial culture was centrifuged, and the supernatant fluid was collected and precipitated with ammonium sulfate. The bacterial culture was centrifuged, and the supernatant was collected and precipitated with ammonium sulfate at the range of 30-60%. The precipitate was resuspended in 50 mM Tris-HCl (pH 8.0) and dialyzed overnight against 20 mM Tris-HCl (pH 8.0). The dialyzed solution was applied to a 3.0 × 15 cm Q-Sepharose column (Pharmacia, Upsalla, Sweden), the column was washed with 20 mM Tris-HCl (pH 8.0) until the A_{280} signal returned to baseline, and the bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer at a flow rate of 2 ml/min. The fractions were tested for activity against *Xanthomonas campestris* pv. *vesicatoria* by spotting 10 μl of each fraction on agar plates and overlaying the plates with 7 ml of 0.7% agar containing 100 μl of a solution of the test bacterium at OD_{600} = 0.4. Active fractions were pooled, desalted, and concentrated with a Centricon 10 concentrator (Millipore, Bedford, MA).

The concentrate was applied to a Mono Q HR 5/5 (Pharmacia) column pre-equilibrated with 20 mM Tris-HCl (pH 8.0). The column was washed with the same buffer and eluted with a linear gradient of 0 to 1.0 M NaCl at a flow rate of 1 ml/min. Fractions corresponding to A_{280} peaks were tested for activity against sensitive strains and concentrated using Centricon concentrators as described above. The final concentrate was applied to a Sephacryl S-200 gel filtration column (Pharmacia) and eluted with 0.15 M NaCl in 20 mM Tris-HCl (pH 8) with a flow rate of 0.5 ml/min. Active fractions were tested and concentrated as described above. This partially purified glycinecin A was used in this study, the glycinecin A activity was quantified as described below.

Quantification of Bacteriocin activity: The lethal activity of bacteriocin suspensions were determined by spot tests. The serial dilutions (twofold) of glycinecin A preparation in sterilized 20mM Tris-HCl (pH 8.0) were made, and 10 μl of each dilution was spotted on LB agar and allowed to dry for 10 min. The plate was overlaid with 7 ml of soft agar (0.7%, wt/vol) containing 0.1ml of the indicator strain (OD_{600}, 0.4), and incubated overnight at 28^{o}C. The bacteriocin titer was defined as the reciprocal of the highest dilution factor that showed inhibition of the indicator strain. The activity was calculated as titer^{-1}×100 and indicated in arbitrary units per milliliter (AU/ml).

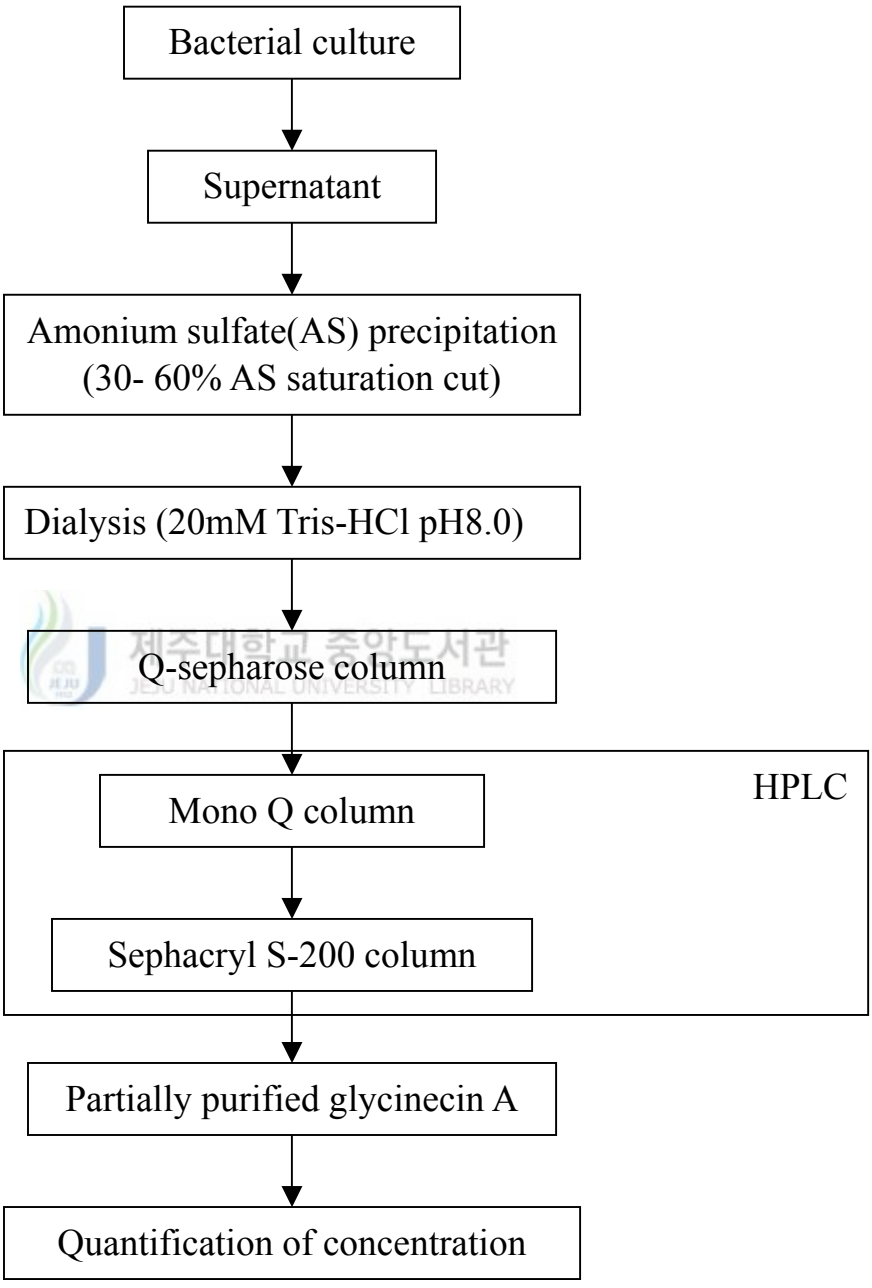
Scheme 1. Schemic diagram of constructing chimeric glycinecin A.



Chimeric glycinecinA

Each gene (*gly*A and *gly*B) was PCR cloned using specially designated primers and ligated together using *Bam*HI restriction site. The fusion gene was ligated into pSGEB through *Eco*RI and *Hind*III sites. The fusion gene contains its own promoter and ribosome binding site. Enzyme abbreviations: H, *Hind*III; Hc, *Hinc*II; RI, *Eco*RI; RV, *Eco*RV; Sm, *Sma*I; Xb, *Xba*I.

Scheme 2. Schemic diagram for the purification of glycinecin A



3. Effect of glycinecin A on the viability of *Xanthomonas* cells

X. campestris pv. *vesicatoria* cells were grown to mid-exponential phase at 28ºC in NB, centrifuged (3,000 rpm, 10 min), washed twice with 20mM Tris-HCl (pH 8.0), and diluted to an OD_{600} of 0.3 (corresponding to 10^8- 10^9 cells/ml) in the same buffer. The cells were then diluted tenfold in 20mM Tris-HCl (pH 8.0) containing glycinecin A at a final concentration of 5120 AU/ml. Samples were taken at the indicated times and diluted 10-10,000-fold in 20 mM Tris-HCl (pH 8.0). Aliquots of 20 μl were spotted in duplicate on NB agar plates for overnight culture at 28ºC, and colony-forming units (cfu) were counted manually.

The relationship between the glycinecin A concentration and its lethal activity on *X. campestris* pv. *vesicatoria* cells was tested as follows. *X. campestris* pv. *vesicatoria* cells were grown with shaking overnight at 28ºC, harvested, washed with 20 mM Tris-HCl (pH 8.0), and resuspended in the same buffer to an OD_{600} of 1.0. Glycinecin A was then added at varying concentrations to 1 ml of the bacterial culture and the mixtures were incubated for 120 min. The cultures were centrifuged, and the pellets were resuspended in 10 ml of fresh nutrient broth and incubated at 28ºC for 10 h in a rotary shaker. The OD_{600} was monitored every 2 to 3 h using a Tecan ELISA reader (Tecan, Austria).

4. Effects of glycinecin A on membrane proton motive force of sensitive cells

Measurement of membrane potential ($\Delta\Psi$): The membrane potential of *X. campestris* pv. *vesicatoria* YK93-4 cells was measured by monitoring the fluorescence intensity of the fluorescent probe $DiSC_3(5)$ as described previously (Herranz *et al.*, 2001a) with the following modifications. The cells were harvested in the exponential phase of growth (OD_{600} = 0.4) by centrifugation (3,000 rpm, 10 min) at 4ºC, washed twice in 50 mM K-HEPES (pH 7.0), resuspended in the same buffer to approximately 1/20 of the original volume, and stored on ice until use. The cell suspension was diluted to a final OD_{600} of 0.3 in 50 mM K-HEPES containing 10 mM glucose and 5 μM $DiSC_3(5)$. The fluorescence intensity was measured with an F-4500 spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan) at 30ºC in a stirred cuvette (excitation wavelength of 643 nm, slide width of 5 nm; emission wavelength of 666 nm, slide width of 5 nm). The ΔpH was depleted by addition of the H/K exchanger nigericin to a final concentration of 5 μM.

After reaching a steady-state ΔΨ, glycinecin A was added to the reaction at a final concentration of 5120 AU/ml. The K ionophore valinomycin (final concentration, 5 μM) was used to obtain control samples with no membrane potential.

Measurement of Membrane pH gradient. The transmembrane pH gradient of target cells was measured by monitoring the fluorescence intensity of the pH-sensitive fluorescent probe BCECF as described previously (Molenaar *et al*., 1991). Briefly, cells were loaded with BCECF by incubating cell suspensions (OD_{600} = 2 in 50mM KPi) with 1mM BCECF and 0,05 N HCl for 5 min. The loading was followed by four rapid washes. The BCECF loaded cells were then diluted to an OD_{600} of 1 and glycinecin A was added to the cell suspension at a final concentration of 5120AU/ml. Measurements were made with an F-4500 spectrophotometer (Hitachi) in a stirred cuvette (excitation wavelength of 502 nm, slide width of 5 nm; emission wavelength of 525 nm, slide width of 10 nm).

5. Measurement of the extracellular K^+, Mg^{2+} and PO_4^{3} ions concentration

The effects of glycinecin A and valinomycin on the efflux of K^+, Mg^{2+} and PO_4^{3-} ions were measured as described previously with slight modifications (Strauch *et al*., 2001). Briefly, a 100-ml culture of *X. campestris* pv. *vesicatoria* cells was grown with shaking at 28ºC to an OD_{600} of 0.6. The cells were harvested, washed three times in 20mM Tris-HCl (pH.8), and resuspended to their original volume in the same buffer. Glycinecin A was then added to different final concentrations of 100, 500 and 5120 AU/ml. Samples were collected at indicated time after the addition of glycinecin A and immediately centrifuged at 12,000 rpm for 5 min, and the supernatants were frozen in liquid nitrogen. In other experiments, valinomycin was used. Negative control samples were not treated with glycinecin A or valinomycin, and positive control samples were heated to 100ºC for 10 min. The concentrations of K^+, Mg^{2+} and PO_4^{3-} ions were determined on an atomic absorbance spectrometer (PerkinElmer 3100; PerkinElmer Optoelectronics, Freemont, CA), and each treatment was performed in triplicate.

III. **RESULTS**

1. Effects of glycinecin A on the viability of sensitive *Xanthomonas* cells

The antibacterial activity of glycinecin A on *X. campestris* pv. *vesicatoria* cells was assayed by incubating the cells with glycinecin A at a concentration of 5120 AU/ml. As shown in Figure 1, the survival of cells decreased as a function of time, diminishing gradually during first 30 minutes of treatment with glycinecin A. survival reduced only about 40%. Survival decreased markedly after 30-120 minutes of incubation, with decreases in viability of 40- to 100 percent compared to negative controls. In contrast, valinomycin at a concentration of 50μM did not kill the cells totally after 120 minutes of treatment, about 60% of cells were still survival.

Figure 2 shows the relationship between the killing activity and the concentration of glycinecin A. A decrease in the glycinecin A concentration led to a decrease in its bactericidal activity during 120 min of treatment. A glycinecin A concentration of 5120 AU/ml inhibited the growth of *X. campestris* pv. *vesicatoria* cells almost completely after 120 min of incubation. However, glycinecin A at concentration of 2560 AU/ml did not kill all of the *X. campestris pv. vesicatoria* cells after 120 min of treatment; and the culture had an OD_{600} of 0.2 after 10 h of recovery incubation with shaking at 28ºC. A further reduction of glycinecin A concentration shows the further increase of the OD_{600} and the lethal activity of the bacteriocin was very low at a final concentration of 320 AU/ml, a recovery OD_{600} of 0.5 was measured.

2. Effects of glycinecin A on the pH gradient (ΔpH) of *Xanthomonas* cells

The fluorescent probe BCECF can be used to measure the transmembrane pH gradient (ΔpH). Changes in the fluorescence intensity of this reagent, due to extrusion or loss of intracellular BCECF, indicate depletion of the membrane pH gradient (Molenaar *et al*., 1991). Glycinecin A treatment caused a marked increase in the fluorescence intensity of BCECF-loaded *X. campestris* pv. *vesicatoria* cells, indicating depletion of the membrane pH gradient of these cells (Fig. 3A). The ΔpH of sensitive cells was dissipated almost completely by glycinecin A treatment, as indicated by a small increase in the fluorescence intensity due to the H^+/K^+ exchanger nigericin, which rapidly reversed the pH of the cells to that of the medium (Herranz *et al*., 2001b). In contrast, glycinecin A did not dissipate the ΔpH of *E. coli* cells (Fig. 3B), although treatment with 5 μM nigericin was able to completely dissipate the pH gradient of these cells. These results suggest that glycinecin A may selectively form pores or break ion channels in *X. campestris* pv. *vesicatoria* cells but not in *E. coli* cells.

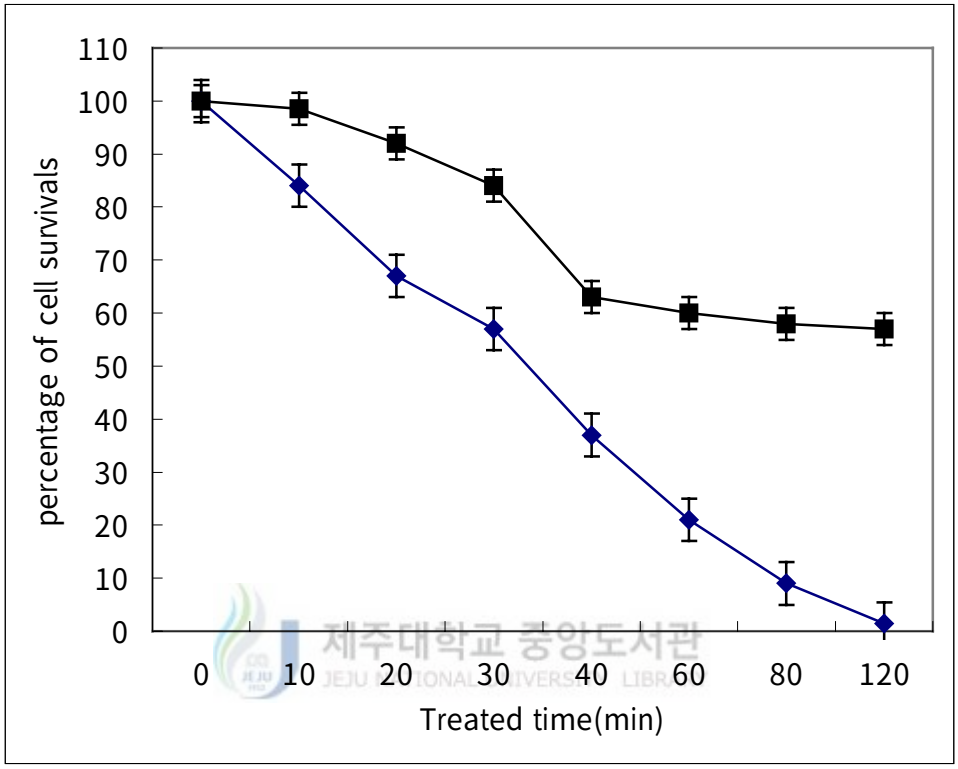


Figure 1. Effects of glycinecin A and valinomycin on the viability of *X. campestris* pv. *vesicatoria* cells as a function of time, survival percentages of *X. campestris* pv. *vesicatoria* cells incubated in 20 mM Tris-HCl (pH 8.0) after exposure to 5120 AU/ml glycinecin A(◆), and with 50μM valinomycin(■). Error bars represent standard deviation of three independent experiments.

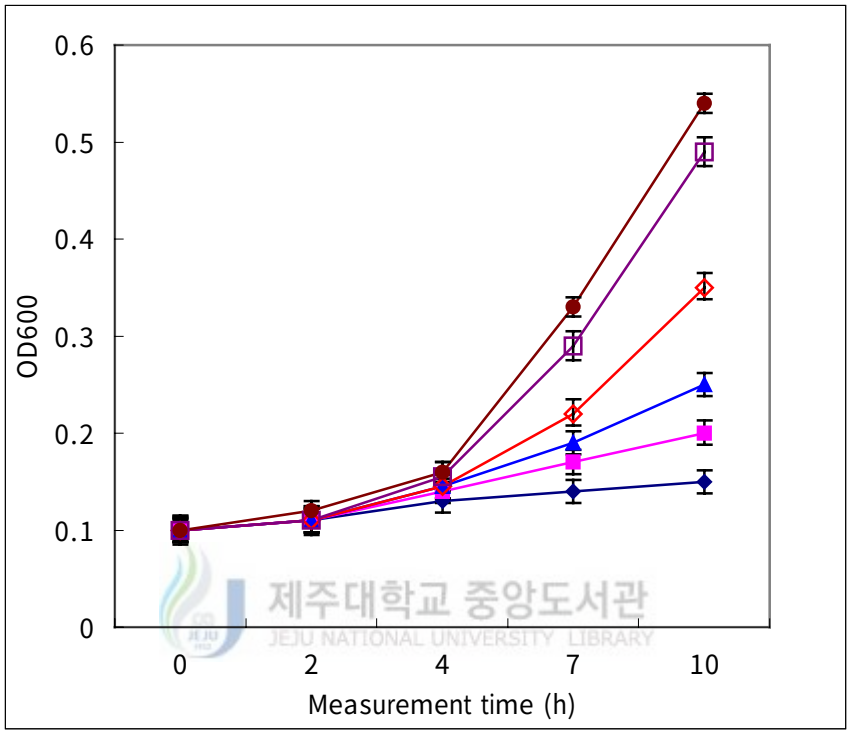


Figure 2. Effects of varying doses of glycinecin A on *X. campestris* pv. *vesicatoria* cells. Glycinecin A was added to a 1-ml sample of overnight culture to final concentrations of 5120 (◆), 2560(■), 1280(▲), 640(◇) and 320(□) or 0 (●) AU/ml, and the cultures were incubated for 120 min. Next, 10 ml of fresh NB were added, the cultures were shaken at 28ºC, and OD_{600} was monitored every 1-2 h, Error bars represent standard deviation of three independent experiments.

3. Effects of glycinecin A on the membrane potential of *Xanthomonas* cells

Many bacteriocins dissipate the transmembrane potential related to active transport by first inducing leakage of ions through the formation of voltage-dependent channels in phospholipid bilayer membranes. This leads to the loss of ions, such as potassium and magnesium, which is the primary cause of cell death. The effect of glycinecin A on the membrane potential of *Xanthomonas* cells was measured using the fluorescence intensity of the potentiometric dye $DiSC_3(5)$. Changes in the fluorescence of the dye indicate the generation or disruption of membrane potential (Breeuwer *et al.,* 2000). In cells energized with glucose, a rapid quenching of fluorescence was observed after addition of the dye (data not shown), indicating the generation of membrane potential ($\Delta\Psi$). The $\Delta\Psi$ of *X. campestris* pv. *vesicatoria* cells was dissipated upon addition of glycinecin A to a concentration of 5120 AU/ml. The increase in the fluorescence intensity of $DiSC_3(5)$ reflected the disruption of the membrane potential. Valinomycin at 5 μM dissipated the membrane potential of *X. campestris* pv. *vesicatoria* cells almost completely after 15 min of treatment. Glycinecin A at a concentration of 5120 AU/ml disrupted the membrane potential of similar cells more gradually than did 5 μM valinomycin (Fig. 4).

Glycinecin A was observed to dissipate the membrane potential further after 20 min of measurement (data not shown). This result implies that the bactericidal action of glycinecin A progresses slowly, as the survival of sensitive cells decreased with increasing incubation time. Membrane potential may be a primary indicator of the further efflux of ions, such as potassium ions, in *X. campestris* pv. *vesicatoria* cells after glycinecin A treatment.

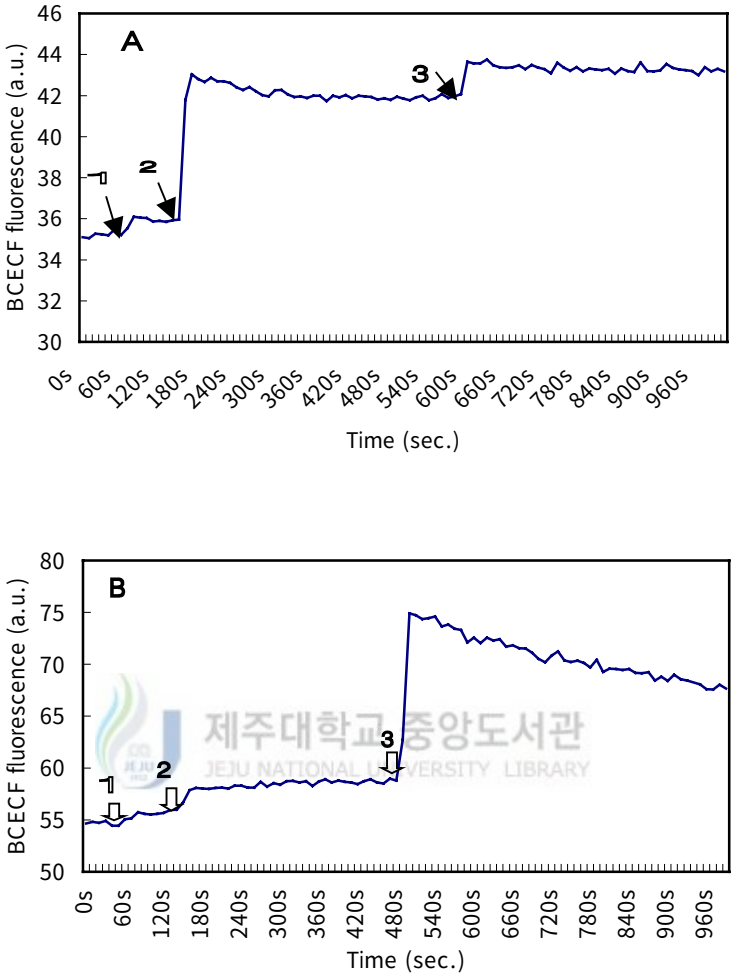


Figure 3. Effects of glycinecin A and nigericin on the ΔpH of X. campestris pv. vesicatoria (A) and E. coli (B) cells, as indicated by the efflux of the fluorescent dye BCECF. BCECF-loaded and glucose-energized cells were diluted in 50 mM potassium phosphate buffer. Valinomycin was added to a concentration of 5 μM at the time indicated by arrow 1 to dissipate ΔΨ, and glycinecin A (5120 AU) and nigericin (5 μM) were added at the times indicated by arrows 2 and 3, respectively. These data represent for three independent experiments. AU, arbitrary units.

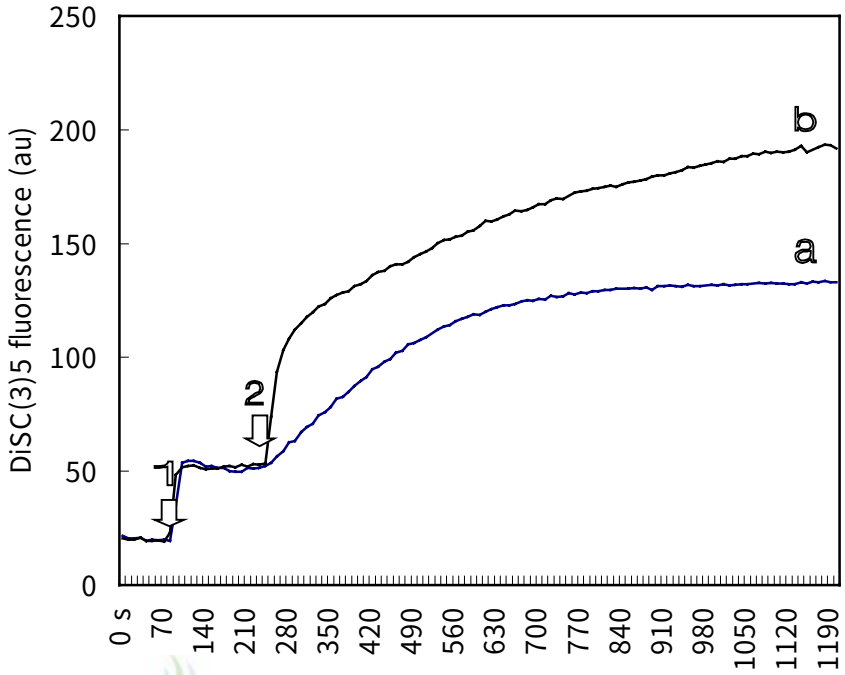


Figure 4. Effects of glycinecin A (a) and valinomycin (b) on the membrane potential of *X. campestris* pv. *vesicatoria* cells, as indicated by the fluorescence intensity of the dye. Cells were energized with 10 mM glucose and labeled with $DiSC_3(5)$. Next, the cell suspension was treated with 5 μM nigericin at the time indicated by arrow 1 to dissipate the transmembrane ΔpH, and valinomycin at 5 μM or glycinecin A at 5120 AU/ml were added at the time indicated by arrow 2. Data represent for three independent experiments. AU, arbitrary units.

4. Effects of glycinecin A on the effluxes of K^+, Mg^{2+}, and PO_4^{3-} ions from *X. campestris* pv. *vesicatoria* cells

To confirm the activity of glycinecin A on *X. campestris* pv. *vesicatoria* cells, the effluxes of K^+, Mg^{2+} and PO_4^{3-} ions were measured as described above. Fig. 5 shows the effluxes of these ions with time after glycinecin A treatment. The total intracellular K^+ ion content of the bacterial suspension (10^8-10^9 cells/ml) was released by boiling, to a concentration equal to 6.4 mg/l. Samples treated with glycinecin A for 10 min displayed slightly changes in extracellular potassium ion concentration as compared to untreated controls. This suggests that the K^+ voltage channels of sensitive cells were non-functional and open after 10 min of incubation with glycinecin A, as indicated by the extracellular K^+ ion concentration, which increased markedly after 10 min, up to 2.9 mg/l after 60 min of incubation, representing nearly half of the total K^+ in the cells. These data demonstrate a strong correlation between the release of K^+ ions and the percentage of surviving cells in cultures incubated for varying times with glycinecin A. The significant increase of K^+ efflux was observed during period of 10 to 60 min of treatment, corresponding to a marked increase of cell death (Fig. 1).

Interestingly, the extracellular concentrations of Mg^{2+} and PO_4^{3-} ions show no changes during the time of the treatment in comparison to their untreated controls. This indicates the increase of extracellular concentration of K^+ is not due to the death of the cells (Fig. 5). Meanwhile, another data show that, different concentrations of glycinecin A (100, 500 and 5000 AU/ml) induced the release of K^+ ions at different levels (corresponding to 1.6, 2.0 and 3.15 mg/l respectively) (figure 6). Lower the concentration of glycinecin A led to smaller amount of K^+ ion efflux from indicator cells after 90 min of incubation. With the reduction of glycinecin A concentration, the effluxes occurred more gradually during that time.

Valinomycin, an ionophore with high specificity for K^+, that can convert a concentration gradient for K^+ of bacterial cells into equilibrium across the membranes (Altendorf *et al*., 1986), can gradually induced the efflux of K^+ ions of *Xanthomonas* cells only after 10 minutes of incubation (Fig.7), even though it can induce very fast induction of

membrane potential of this cells (Fig.4). The further reduction of valinomycin concentration, the longer time needed for induction of K^+ leakage. But, the difference of the extracellular K^+ concentrations between two treatments of 5µM and 50µM valinomycin was not significant after 80 min of incubation (1.8 and 2.04 mg/l respectively), and these concentrations of K^+ releases are somehow equal to that released due to glycinecin A at a concentration of 500au, that did not kill totally the cells (figure 2).

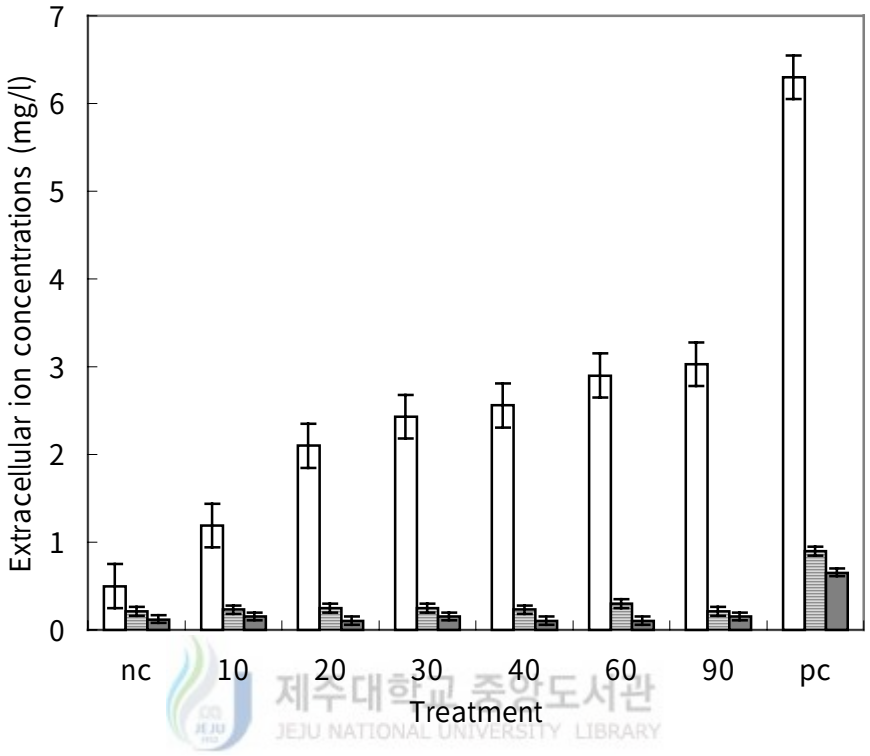


Figure 5. Effects of glycinecin on the effluxes of K^+, Mg^{2+} and PO_4^{3-} ions. Indicator cells were incubated with glycinecin A at a final concentration of 5120 AU/ml and samples were taken at the indicated times to measure the extracellular K^+ (white), Mg^{2+}(paralleled lines) and PO_4^{3-} (gray) ion concentrations. NC: negative control, PC: positive control. Error bars indicate standard deviation of three independent experiments.

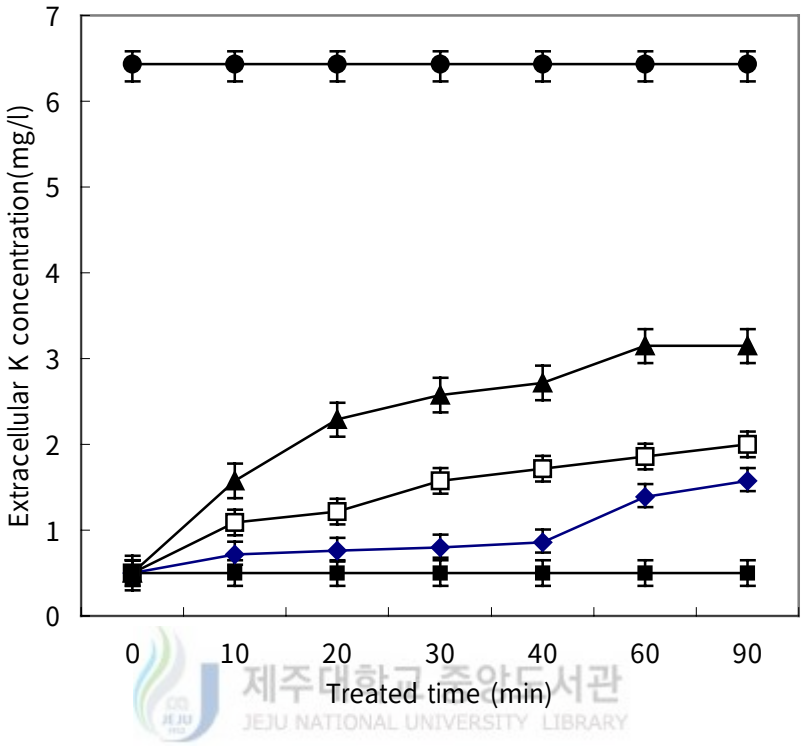


Figure 6. Effect of glycinecin A concentration on K^+ ion efflux. Indicator cells were incubated with different concentrations of glycinecin A 100AU/ml (♦), 500 AU/ml (□) or 5000 AU/ml (▲), Negative control (■) and positive control (●). The extracellular K^+ concentrations were measured at indicated times. Error bars indicate standard deviation of three independent experiments.

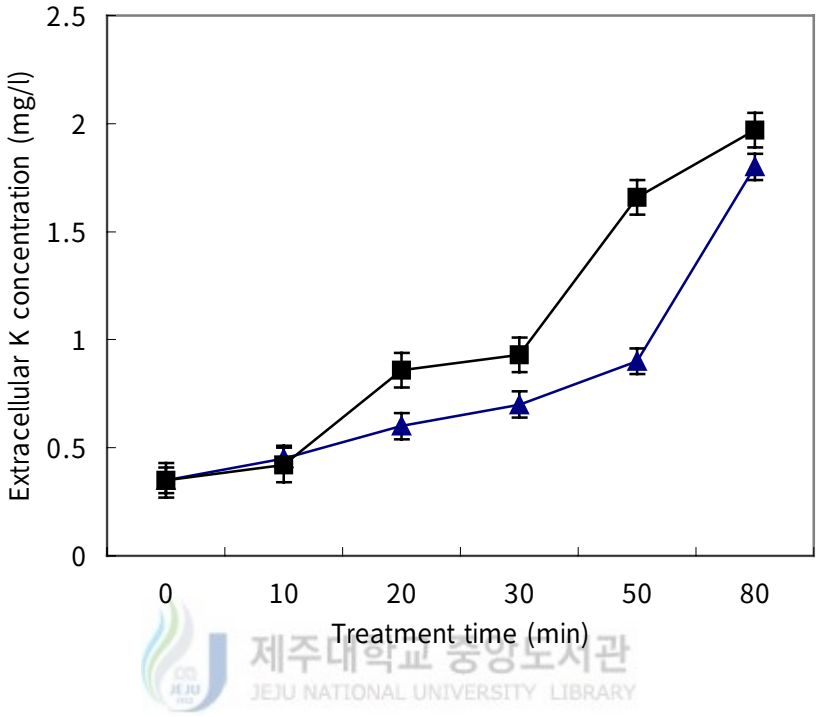


Figure 7. Effect of valinomycin on K^+ ion efflux: Indicator cells were incubated with valinomycin at different concentrations: 5μM (▲) and 50μM (■), positive control K^+ concentration is equal to 6.2 mg/l, negative control is equal to 0 min. Error bars indicate standard deviation of three independent experiments.

IV. DISCUSSION

In general, pore-forming bacteriocins have lethal activity because they alter the permeability of the cell membrane, disrupting the membrane proton motive force and the efflux of ions, such as potassium, magnesium, sodium, and chloride (Daw *et al.,* 1996, Herranz *et al* 2001a and 2001b; Moll *et al*., 1996). As with other known two-component bacteriocins, including the plantaricin A system, plantaricin S, lactococcin S and lactococcin M, which cause the efflux of intracellular potassium ions, the dissipation of proton motive force leads to the hydrolysis of ATP in susceptible bacteria (Moll *et al*., 1996). Glycinecin A also induces the dissipation of membrane potential (ΔΨ), depletion of the pH gradient, and efflux of potassium ions. Plantaricin JK and EF disrupt the proton motive force (ΔΨ and ΔpH) of sensitive cells. However, ΔpH depletion is more effective in inhibiting cell growth than the dissipation of ΔΨ. The depletion of the pH gradient leads to a drop in the intracellular pH and subsequent inhibition of cellular metabolism (Moll *et al.,* 1999). Our results indicate that glycinecin A causes slow dissipation of ΔΨ but rapid depletion of ΔpH. This suggests that glycinecin A inhibits the growth of sensitive cells by the dissipation of ion channels, leading to efflux of hydroxyl ions followed by leakage of potassium ions. The antimicrobial activities of lactococcins A, B and G and other class II bacteriocins are thought to require a receptor-like factor (Herranz *et al.,* 2001a). The disruption of the pH gradient of *X. campestris* pv. *vesicatoria* cells by glycinecin A, which is not observed with *E. coli* cells, may be due to a specific receptor that is present in the former cells but not the latter.

The circulation of potassium ions is important in several homeostatic mechanisms, such as the regulation of intracellular pH and osmotic strength. Electrogenic potassium efflux causes the collapse of ΔΨ, followed by inhibition of the uptake of amino acids. Release of potassium ions causes an increase in ATP hydrolysis by FoF_1-ATPase in the cytoplasm of sensitive strains (Moll *et al.,* 1999). The loss of intracellular K^+ and the dissipation of proton motive force (ΔΨ and ΔpH) cause ATP depletion but not ATP efflux, leading to disruption of the active transport of organic and inorganic molecules into and out of the cell, which causes the death of susceptible strains (Herranz *et al.,* 2001a; Minahk *et al.,* 2000; Moll *et al.,* 1999). On the basis of ion selectivity, two-

peptide bacteriocins are divided into two subgroups; the first contains monovalent cation-conducting systems, such as lactococcin and plantaricin EF, while the second contains bacteriocins with a preference for anions, such as plantaricin JK and possibly acidocin J1132 (Moll *et al.,* 1999). Glycinecin A treatment caused slight leakage of cations, such as K^+ and H^+, as indicated by depletion of the pH gradient and increases in the extracellular potassium ion concentration following its addition. Glycinecin A causes slow efflux of potassium ions, and its lethal action is strictly dose-dependent. The induction of K^+ ion leakage strictly causes the death of *Xanthomonas* cells, total cell death occurs only when the efflux of K^+ ion concentration is about half of total that of the cells as indicated by treatment of the cells with glycinecin at a final concentration of 5120AU/ml (Fig. 2 and 6), and the lower the leakage of K^+ ions shows the lower number of dead cells, as indicated by the relationships between viabilities of cells and the K ion effluxes caused by glycinecin A at a final concentration of 500AU/ml (Fig. 2 and 6) and valinomycin at a final concentration of 50μM (Fig.1 and 7). Pore-forming peptides may cause the efflux of macromolecules, such as β-galactosidase (Hisham *et al.,* 2000; Silvestro *et al.,* 2000), and the release of A_{280}-absorbing materials (Minahk *et al.,* 2000). Our studies indicate that glycinecin A does not cause the efflux of macromolecules, such as β-galactosidase, or induce the release of A_{280}-absorbing materials (data not shown) from sensitive cells, which suggests that release of K^+ is not due to the cell death.

In conclusion, the bactericidal activity of glycinecin A on *X. campestris* pv. *vesicatoria* cells may depend on the breakage of voltage channels, which leads to the efflux of potassium ions and dissipation of the membrane potential and pH gradient. The time-dependent killing activity and the release of potassium ions observed in this study indicate that potassium ion leakage occurs simultaneously with cell death. Meanwhile the bacteriocin did not cause the release of Mg^{2+} and PO_4^{3-} ions. These suggests that potassium leakage is the main cause of death of *X. campestris* pv. *vesicatoria* cells triggered by glycinecin A treatment.

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초 록

Xanthomonas campestris pv. glycines 8ra에 의해 생성된 박테리오신의 일종인 glycinecin A가 인접한 다른 세균의 생육을 억제한다는 사실은 과거 Heu (Heu *et al*., 2001)에 의해 증명되어졌다. 이 논문에서 우리는 세포의 치사를 야기하는 glycinecin A의 특성을 알아보고자 한다. glycinecin A는 대표적인 세포막전위 분해제인 valinomycin에 비해 막전위의 유실 속도가 더 느렸다. 이에 반해 *Xanthomonas* 균에서의 pH 농도구배의 변화는 H^+/K^+ 교환체인 nigericin 만큼 신속히 유도하였다. 반면, glycinecin A는 *E. coli*의 pH 변화를 야기하지는 않았다. 이는 glycinecin A의 결합과 활성을 위해서는 특이 수용체가 필요함을 보여준다. glycinecin A를 *X. campestris* pv. vesicatoria YK93-4 균에 처리했을 때, 세포로부터 칼륨 이온의 누출 역시 유도되어졌지만, 그 외에 마그네슘과 같은 다른 이온의 유실은 일어나지 않았다. Glycinecin A 에 의한 이러한 칼륨 이온의 유실은 valinomycin에 비해 더 빠르고 더 많이 일어났다. Bacteriocin이 투여된 세포는 배양 2시간내에 죽었고, 대부분의 세포는 glycinecin A에 의해 야기된 칼륨 이온의 손실에 기인하였다. 이러한 결과들은 glycinecin A의 활성 기작이 수소와 칼륨 이온의 막 투과에 밀접하게 관련되어 있음을 보여준다.

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