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# Improvement in the Stability of Glycinecin A through Protein Fusion of the Two Structural Components

**Youngmee Kim, Somi K. Cho, and Moonjae Cho***

*Department of Biochemistry, Cheju National University Medical School, Jeju 690-756, Korea*

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**Glycinecin A, a bacteriocin produced by *Xanthomonas campestris* pv. *glycines*, inhibits the growth of *X. c.* pv. *vesicatoria*. We have reported that purified glycinecin A is composed of two polypeptides, is active over a wide range of pH (6 to 9), and is stable at temperatures up to 60°C. Glycinecin A is a heterodimer consisting of 39- and 14-kDa subunits; the two encoding genes, *glyA* and *glyB*, respectively, have been cloned (Heu *et al.* 2001. *Appl. Environ. Microbiol.* 67, 4105-4110). Co-expression of *glyA* and *glyB* in the same cell is essential for bacteriocin activity. We constructed and produced a chimeric glycinecin A connecting *glyA* and *glyB* in one open reading frame. The chimeric glycinecin A has the same bactericidal activity as the wild-type glycinecin A. However, the chimeric glycinecin A is more stable in a wider range of pH and temperature.**

***Key words***: glycinecin, chimeric protein stability, *Xanthomonas*, bacteriocin

Bacteriocins are bactericidal compounds, usually proteinaceous, whose activity is often restricted to bacterial strains that are closely related to the bacterium they are produced from (6, 21). Their production occurs across all major groups of Eubacteria and Archaebacteria (20). Some bacteriocins, such as halocins from Halobacteria, have no protein sequence homology to known bacteriocins (18). Others, such as thepyocins of *Pseudomonas aeruginosa*, some of the colicins from *E. coli*, and a cloacin of *Enterobacter cloacae* reveal protein sequence homology (13, 14, 17).

Among phytophathogenic bacteria, bacteriocins have been reported from *Agrobacterium radiobacter* (15), *Corynebacterium ulcerans* (1), *Pseudomonas syringae* (19), *Erwinia carotovora* subsp. *carotovora* (3), *and X. campestris* (4). The bacteriocin activity from *Xanthomonas campestris* pathovar (pv.) *glycines* was first reported by Fett *et al.* (4) and named glycinecin. Heu *et al.* (5) reported the cloning of the gene encoding glycinecin A from *X. campestris* pv. *glycines*. It was found to consist of two structural genes (*glyA* and *glyB*) which encode 39-kDa and 14-kDa proteins respectively.

Glycinecin A shows selective bactericidal activity toward pathogenic bacteria originating from *Xanthomonas* species such as *X. campestris* pv. *vesicatoria, X. campestris* pv. *campestris*, and *X. oryzae* pv. *oryzae* in green house and field applications (7). Glycinecin A has ideal properties of agents for controlling xanthomonad pathogens, such as high specificity, low cost, and safety for the user and the environment. However, its chemical composition and structure, mode of action, and stability remain to be investigated.

We reported that the bactericidal activity of glycinecin A depends upon the formation of heterodimer and its stability improves when the two subunits are chemically cross-linked. Here we generated a chimeric fusion protein, chimeric glycinecin A, in which two subunits are connected to produce one polypeptide through genetic engineering. Its bacteriocin activity is the same as the wild type protein but its stability against temperature and pH is improved.

## Materials and Methods

***Construction of a chimeric glycinecin A***

We designed a chimeric protein that combines *glyA* and *glyB* in one protein. Fig. 1 shows the schematic diagram of the generating fusion protein. *glyA* was cloned by polymerase chain reaction (PCR) using a 5'primer containing the *Hin*dIII restriction enzyme site and a 3'primer containing the *Bam*HI site instead of a stop codon, respectively (5'primer; CCCTGGCCGTCTCGCCGCCGTACAAGCT TAATG, 3'primer; AAGGATCCGGGCTCGGCTCGGC ATACCTTTCCTCC). *glyB* was also PCR-cloned using a 5'primer containing the *Bam*HI restriction site instead of the ATG codon and a 3'primer containing the *Eco*RI site after the stop codon (5'primer; AAGGATCCGGCGCCCG-TGCACAGAACGACG, 3'primer; CCGAATTCCTGAAA CTTTAGTGCAATAGATCTACGG). Then, the two PCR

* To whom correspondence should be addressed.
(Tel) 82-64-754-3837; (Fax) 82-64-725-2593
(E-mail) moonjcho@cheju.cheju.ac.kr

products were ligated through the same the *Bam*HI site to produce a chimeric glycinecin A and cloned in the bacterial cloning vector. We named this vector pGly415.

***Bacteriocin activity assay***

Bacteriocin activity was determined by examining inhibition zones created on indicator strain, *X. campestris* pv. *vesicatoria* Ds94-9 (11). Cell-free culture supernatants or cell extracts of *E. coli* transfected with pGly415 or pSGEBI were serially diluted (two-fold) in sterile water. A 10-μl aliquot of each sample was then spotted onto LB agar plates and allowed to dry for 10 min. The plate was overlaid with 7 ml of soft agar (0.7%, w/v) containing 0.1 ml of the indicator strain ($O.D_{600}$= 0.1) and incubated overnight at 30°C. Bacteriocin titer was defined as the reciprocal of the highest dilution factor that showed inhibition on the indicator strain. The activity was defined as titer$^{-1}$ × 100, and indicated in arbitrary units per ml (AU/ml). When formation of a turbid zone followed formation of a clear inhibition zone, the critical dilution was taken to be the average of the final two dilutions. To evaluate the stability of glycinecin A, wild-type glycinecin A and the chimeric glycinecin A were incubated at different temperatures for 15 min or in different pH buffers for 30 min and spotted onto agar plates as described above.

***Localization of the recombinant glycinecin A***

*E. coli* cells expressing the wild-type or the chimeric glycinecin A were incubated in a 15-ml test tube containing 5 ml of LB medium at 29°C for 21 h with aeration. Cells were harvested by centrifugation at 4,000 × *g* for 15 min, and the supernatant was analyzed for secreted bacteriocin activity. The cell pellet was resuspended in 30 ml of osmotic shock buffer (30 mM Tris-HCl pH 8.0, 20% sucrose) and 60 μl of 0.5 M EDTA, pH 8.0 was added before incubating the cell suspension at room temperature for 10 min. Cells were collected by centrifugation at 10,000 × g at 4°C for 10 min and the pellets were resuspended in 30 ml of ice-cold 5 mM $MgSO_4$ solution and stirred slowly for 10 min on ice. The cell suspension was then centrifuged at 10,000 × g for 10 min and the cell-free supernatant was used for analysis of periplasmic proteins (9). The remaining pellets were suspended in lysis buffer (phosphate-buffered saline containing 1% Triton X-100), sonicated (5 cycles of 20 sec bursts at 300 W at 1 min intervals), and then centrifuged at 10,000 × g for 20 min. The resulting supernatant was used as the cytoplasmic fraction (9).

***Expression of the chimeric glycinecin A***

The chimeric glycinecin A was expressed in *E. coli* transformed with pGly415 (Fig. 1). *E. coli* carrying pGly415 was grown at 37°C in LB containing ampicillin (50 μg/ml). Bacterial cells were harvested through centrifugation and suspended in lysis buffer (0.1 M phosphate buffer containing 1 mM phenylmethylsulfonylfluoride). The cells were sonicated on ice in 1-min bursts at 250 W at 1 min intervals, and the cell extracts were precipitated with ammonium sulfate (30-60%). The final precipitate was resuspended in 50 mM Tris-HCl (pH 8.0) and dialyzed against 20 mM Tris-HCl (pH 8.0) overnight and used for characterization of the chimeric glycinecin A.

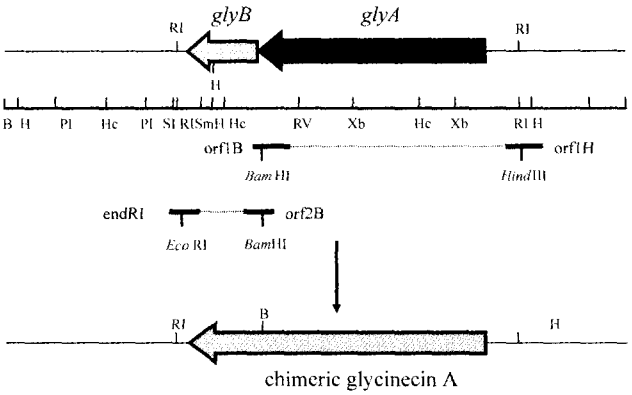


**Fig. 1.** Schematic diagram of constructing chimeric glycinecin A. Each gene (*gly*A and *gly*B) was PCR cloned using specially designed primers and ligated together using the *Bam*HI restriction site. The fusion gene was ligated into pBlueScript through the *Eco*RI and *Hind*III site. The fusion gene contains its own promoter and ribosome binding site. Enzyme abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hind*III; PI, *Pst*I; Hc, *Hinc*II; SI, *Sal*I; Sm, *Sma*I; RV, *Eco*RV; Xb, *Xba*I.

## Results and Discussion

***Construction and expression of a chimeric glycinecin A***

It has been reported that glycinecin A is a heterodimer consisting of 14-kDa and 39-kDa subunits and co-transformation of the two encoding genes *gly*A and *gly*B in the same host is essential for its bactericidal activity (5). The bactericidal activity of glycinecin A was relatively stable in a broad range of temperatures and pHs. However, when the two subunits of glycinecin A are covalently cross-linked by cross-linking agents (DSS), its stability against temperature increased dramatically (5). These results suggest that heterodimer formation is critical for bactericidal activity of glycinecin A and dissociation of the two subunits may be detrimental to its stability. Therefore, a genetically modified chimeric glycinecin A was designed to improve its stability (Fig. 1). Two genes for glycinecin A (*gly*A and *gly*B) were cloned by PCR and connected together in one open reading frame using the same *Bam*HI restriction sites. The fased gene for the chimeric glycinecin A was ligated into pBluescript using the *Eco*RI and *Hind*III sites. As shown in Table 1, wild-type and chimeric glycinecin A have the same bactericidal activity (AU/μg of total protein). Also, most of the expressed chimeric glycinecin A was localized to the periplasmic space as in the case of the wild-type glycinecin A.

**Table 1.** Bacteriocin activity and distribution of chimeric and wild glycinecin A in cells

| Strains (Plasmid) | Proteins | Fractions (AU/μg) | | | |
|---|---|---|---|---|---|
| | | Supernatant | Periplasmic extract | Cytoplasmic extract (soluble protein) | Cytoplasmic extract (insoluble protein) |
| *E. coli* DH5α (pSGEB1) | Glycinecin A | 92.3 | 1316.0 | 315.1 | 148.5 |
| *E. coli* DH5α (pGly415) | Chimeric glycinecin A | 204.1 | 1263.3 | 320.5 | 69.6 |

Both genes (*glyA and glyB*) contained a signal sequence for secretion. It was designed that when the two genes were fused, the signal sequence of *glyB* was located in the middle of the fusion peptide. We designed it like this because the sequence can act as a linker peptide. Now we are constructing several fusion proteins with different sizes of the linker peptides. Since most of the bactericidal activity of the chimeric glycinecin A was found in the periplasmic space, the hydrophobic signal sequence does not seem to affect the translocation of the chimeric glycinecin A through the periplasmic membrane.

### *Stability of the chimeric glycinecin A*

Covalently cross-linked glycinecin A exhibits higher stability at higher temperatures. This result can be interpreted either as inter-subunit cross-linking reduces the dissociation of the heterodimer or as the intra-molecular cross-linking stabilizes the conformation of each subunit. Chimeric or wild-type glycinecin A was treated with described temperature for 15 min prior to inhibition zone assay. Inhibition zone assay was performed with serially diluted samples against the indicator strain. Fig. 2 (insert) clearly shows that there is no difference in bacteriocin activity between the two samples when they were pretreated at 50°C. However, at higher temperatures, only the chimeric glycinecin A shows a clear zone. The stability of the fusion protein at higher temperatures is illustrated in Fig. 2.

Chimeric glycinecin A also showed the bacteriocin activity in a wider range of pH compared to the wild-type

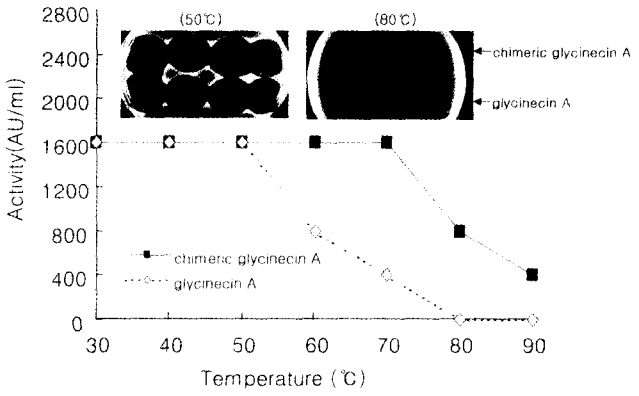


**Fig. 2.** Comparison of thermal stability of wild and chimeric glycinecin A. Inhibition zone assays of serially diluted wild and chimeric glycinecin A were performed after heat treatments. The clear zone after 50 and 80°C treatment are shown in the insert.

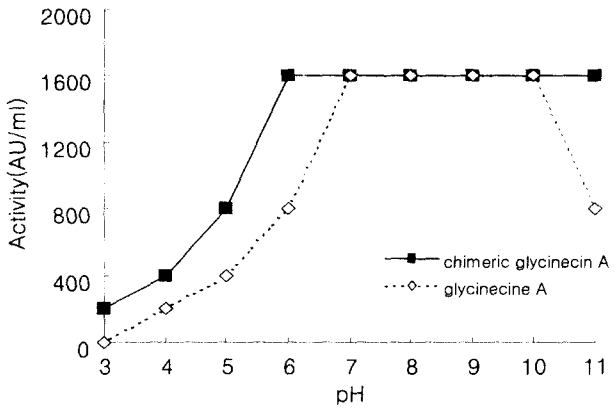


**Fig. 3.** Comparison of the levels of pH dependence of the stability of wild and chimeric glycinecin A. Serially diluted wild and chimeric glycinecin A were incubated for an hour in indicated pH buffers prior to inhibition assay.

glycinecin A. Glycinecin A was incubated in various pH buffers for an hour and tested for inhibition assay. Chimeric glycinecin A was active at lower and higher pH where wild-type glycinecin A does not have the activity (Fig. 3). These results imply that heterodimer formation is again important for the stability of glycinecin A.

Some bacteriocins require the complementary action of two different peptides to achieve their biological activity; these two-peptide groups include lactacin F (2), lactococcin G (12), and cytolysin L (8). The complementary action of bacteriocins is rather common among those isolated from gram-positive bacteria, but bacteriocins from gram-negative bacteria are rarely multimeric. It has not been tried to make a fusion peptide to improve its activity or stability in two-peptide group bacteriocin. However, Rollema *et al.* (1995) changed the amino acid to improve solubility and stability of nisin. Its bactericidal activity was found to be similar whereas its solubility at pH 7 increased by factor 4.

For the application of bacteriocins as biological control agents, stability of the peptides is one of the important factors to be considered because the formulation process usually requires harsh conditions for proteins and inactivation of proteins by denaturation is a common problem during storage. The results of the present study provide information on improving the stability of glycinecin A. The fusion protein also can be used when glycinecin A is applied to generate genetically modified plants.

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