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Characteristics and Lytic Activity of Phage-Derived Peptidoglycan Hydrolase, LysSAP8, as a Potent Alternative Biocontrol Agent for *Staphylococcus aureus* S

Jun-Hyeok Yu[1], Jeong-A Lim[2], Hyun-Joo Chang[2*], and Jong-Hyun Park[1*]

[1]*Department of Food Science and Biotechnology, College of BioNano Technology, Gachon University, Seongnam 13120, Republic of Korea*
[2]*Research Group of Consumer Safety, Korea Food Research Institute, Wanju-gun 55365, Republic of Korea*

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*Corresponding authors
J.H.P.
Phone: +82-31-750-5523
E-mail: p5062@gachon.ac.kr
H.J.C.
Phone: +82-63-219-9326
E-mail: hjchang@kfri.re.kr

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Outbreaks of staphylococcal food poisoning (SFP) causing serious human diseases and economic losses have been reported globally. Furthermore, the spread of *Staphylococcus aureus* with increased resistance to multiple antimicrobial agents has become a major concern in the food industries and medicine. Here, we isolated an endolysin LysSAP8, as one of the peptidoglycan hydrolases, derived from the bacteriophage SAP8 infecting *S. aureus*. This endolysin was tagged with a 6×His at the C-terminal of the target protein and purified using affinity chromatography. LysSAP8 demonstrated lytic activity against a broad spectrum of bacteria, which included a majority of the staphylococcal strains tested in this study as well as the methicillin-resistant *S. aureus* (MRSA); however, no such activity was observed against other gram-positive or gram-negative bacteria. Additionally, LysSAP8 could maintain bactericidal activity until 0.1 nM working concentration and after heat treatment at 37°C for 30 min. The ability of LysSAP8 to lyse cells under varying conditions of temperature (4–43°C), pH (3–9), and NaCl concentrations (0–1,000 mM), and divalent metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, and Zn^{2+}) was examined. At the optimized condition, LysSAP8 could disrupt approximately 3.46 log CFU/ml of the planktonic cells in their exponential phase of growth within 30 min. In this study, we have suggested that LysSAP8 could be a potent alternative as a biocontrol agent that can be used to combat MRSA.

Keywords: *Staphylococcus aureus*, bacteriophage, endolysin, MRSA

Introduction

Staphylococcus aureus is a gram-positive, facultative, anaerobic bacterium commonly found in households and communities such as schools, farms, and workplaces, and on the human skin [1]. These bacteria possess multiple virulence factors that cause the rapid onset of various symptoms of foodborne diseases such as nausea and violent vomiting with or without diarrhea [2]. Many outbreaks of food poisoning caused by *S. aureus* have been reported globally, including in the Republic of Korea, Europe and the United States [3−5]. Unfortunately, *S. aureus* not only causes economic losses in the food industry, but also contributes to the spread of multidrug-resistant (MDR) infections, and strains of methicillin-resistant *Staphylococcus aureus* (MRSA) are the common drug-resistant types that have been identified [6]. Although most of the MRSA strains are associated with infections of the skin and soft tissues, approximately 5−10% cause life-threatening conditions like septicemia and necrotizing pneumonia [1].

The emergence of multidrug-resistant bacteria has led to the development of therapies employing bacteriophages as alternative options to treat these infections [7, 8]. The applications of bacteriophages in clinical therapy, displaying, food safety, and decontamination have been researched globally [9]. Although the use of phages has

seen a number of achievements, several concerns regarding their safety, efficacy, and development of bacterial resistance towards phages still exist [10]. Consequently, there has been an increased focus on the application of the endolysin derived from bacteriophage [11−13]. Endolysins are responsible for the enzymatic degradation of the host peptidoglycan (PG) resulting in the release of progeny virions and death of the host cell [13]. In several studies, the exogenous application of endolysin as a therapeutic agent has enabled the rapid and successful control of target pathogens both in vitro and in vivo [14−16]. Furthermore, synthetic approaches by chimeric modification of endolysins have successfully enhanced their lytic activities [17]. LysK, as the most well-studied *Staphylococcus* phage endolysin, harbors an N-terminal cysteine/histidine-dependent amidohydrolase/peptidase (CHAP) domain, a central amidase domain, and a C-terminal SH3b cell wall-binding domain [18−21]. LysK-like endolysins have been studied in a broad range of areas such as the food industry, therapy, and protein crystallization [20, 22, 23]. Especially, SAL200 and LysGH15 as endolysins against *Staphylococcus* species exhibited tremendous lysis activities and synergic availabilities against *S. aureus* including MRSA [16, 24]. Nevertheless, only a few LysK-like endolysins have been isolated and characterized, though their potential as available agents for application is unclear.

We have characterized an endolysin designated LysSAP8 as a newly isolated endolysin from *S. aureus*-specific phage SAP8. LysSAP8 is a LysK-like lysin composed of two lytic domains and a single cell wall-binding domain. Many factors that affected the lytic activity of LysSAP8 were examined and its host spectrum was confirmed. The current data validate the potential of LysSAP8 as a promising alternative agent for the biocontrol of *S. aureus*.

Materials and Methods

Bacterial Strains and Culture Conditions

In total, 116 strains were used (Table S1) for the study on the lytic spectrum of LysSAP8. Among the 75 isolates of *S. aureus* that were used, 32 clinical isolates were donated by Severance Hospital (Korea) and the remaining 43 were isolated from food samples between 2005 and 2006 in our laboratory. Type strains were obtained from four microbiology collections (KCTC, KCCM, ATCC, and NCCP) and wild-type strains were donated by the laboratory of professor Young-Seo Park (Gachon University, Korea). All bacterial strains were routinely cultured overnight in Luria-Bertani (LB, Difco Co., USA) broth at 37°C under conditions of shaking, or in LB containing 1.5% agar and incubated at 37°C.

Plaque Assay

In 2013, *S. aureus* KCCM 12103 was used to isolate SAP8 using conventional methods [25]. To confirm the presence of phage plaques, phage lysate of SAP8 was serially diluted in SM buffer (5.8 g/l NaCl, 2 g/l $MgSO_4$, 40 ml/l of 1 M Tris-HCl, 0.1 g/l gelatin, pH 7.5), and overlaid with LB soft agar (0.6% agar) on cells of the host bacterium *S. aureus* KCCM 12103 using the agar layer method. The plate overlaid with agar was incubated overnight at 37°C and the plaque was measured.

Electron Microscopy

The phage lysate was purified using a modified polyethylene glycol precipitation method [26]. Purified phage solution was deposited onto formvar-carbon 200 mesh TH copper grids (Ted Pella, Inc., USA) stained with 2% uranyl acetate. The morphology of SAP8 was examined using a transmission electron microscope (Tecnai G2 F30 S-TWIN, FEI, USA).

Complete Genome Sequencing

Genomic DNA of the phage was extracted using the Genomic DNA Extraction Kit (Qiagen Tissue and Blood Kits, USA), according to the manufacturer's instructions. Purified whole genome of the phage was sequenced using the GS Junior Sequencing system (Roche, USA), and its open reading frames (ORFs) were predicted using Glimmer version 3.02 by Chunlab Inc. (Korea).

Accession Number

The genome of SAP8 was submitted to GenBank under accession number MK801680.

Genomic Analysis of SAP8

The predicted ORFs were annotated using Blastp based on the e-value < 1E-05, and classified into six groups based on their functions: packaging, structure, host lysis, DNA manipulation, DNA regulation, and additional function. The G+C content was predicted using the DNA/RNA GC content calculator from ENDMEMO (http://www.endme mo.com/bio/gc.php), and the tRNAscan-SE 1.21 (The Lowe Lab, University of California Santa Cruz, USA) was used to search for the tRNA [27, 28].

Bioinformatic Analysis of LysSAP8

The modular organization of the bacteriophage endolysin was predicted using information from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Blastp [29]. For phylogenetic analysis, 24 sequences of endolysins, including that of LysSAP8 derived from the bacteriophages infecting *S. aureus*, were aligned using ClustalW [30]. These endolysin alignments were constructed into a phylogenetic tree by the neighbor-joining method with 1000 bootstrap replicates using MEGA 7 (MEGA) [31]. The NCBI database was accessed to identify all endolysin sequences.

Protein Expression and Purification

For purification of the endolysin, the gene encoding LysSAP8

was amplified by PCR using the primers containing 5′ NdeI and 3′ XhoI restriction sites. Based on the putative endolysin gene of SAP8, the primers SAP8_F #5 (5′-GGAATTCCATATGTTAAT GACAAAA-AATCAA-3′) and SAP8_R #4 (5′-CCGCTCGAGAAT CGTGCTAAA-3′) were designed. The amplified PCR product was digested with NdeI and XhoI, and ligated into the pET23a vector containing a C-terminal 6× histidine. The recombinant vector, pET23a_*LysSAP8*, was transformed into competent *Escherichia coli* Rosetta (DE3) cells containing pRARE as a codon supplement [32].

The transformant was grown until this reached an $O.D_{600}$ of 0.7–0.8 in LB broth supplemented with ampicillin (final conc. 50 μg/ml) and chloramphenicol (final conc. 12.5 μg/ml), and induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 18°C for 18 h with shaking [33, 34]. The induced *E. coli* were harvested by centrifugation (4,000 ×*g* for 10 min), resuspended in lysis buffer (50 mM Tris-HCl/500 mM NaCl/10 mM imidazole, pH 8.0), and sonicated on ice for 30 min (5/2 s pulse on/off, Amp 40%) using a model VCX130 device (Vibra-Cell, USA). The debris of lysate was removed by centrifugation (25,000 ×*g* for 10 min) to obtain a soluble form of the endolysin, which was purified by affinity chromatography using an Ni-NTA Superflow resin (Qiagen), according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to identify the purified endolysin and its concentration was determined using the Bradford reagent (Bio-Rad Laboratories, USA) according to the manufacturer's instructions.

Characterization of LysSAP8

The minimal bactericidal concentration (MBC) of LysSAP8 was determined by serially diluting the endolysin [35]. *S. aureus* KCCM 12103 as a target bacteria of the endolysin was cultured to its exponential phase (>10^8 CFU/ml) and washed twice with the reaction buffer (50 mM Tris-HCl/500 mM NaCl, pH 8.0) by centrifugation (4,000 ×*g* for 10 min). LysSAP8 was serially diluted and inoculated into the washed *S. aureus* (final conc. 1,000, 100, 10, 1, 0.1, 0.01 nM) culture. After incubation at 30°C for 30 min, the $O.D_{600}$ value was determined. The MBC was defined as the lowest concentration generating turbidity reduction of the target bacteria at 30°C for 30 min.

The range of lysis activity of LysSAP8 was tested against 116 bacterial strains including MRSA by the spot assay [24]. LysSAP8 was spotted onto the bacterial lawn, the cells of which were in their exponential phase of growth and dried at room temperature for 30 min. The spotted plates were incubated overnight at 37°C and observed for the formation of clear zones.

To assess the thermal stability of LysSAP8, LysSAP8 was pre-incubated at high temperatures (30°C, 37°C, 40°C, 45°C, 50°C, 60°C, 70°C, and 80°C) for 30 min [36]. Untreated endolysin served as the control. The treated LysSAP8 was inoculated onto the cell preparations. After incubation at 30°C for 30 min, its residual activity was measured by determining the $O.D_{600}$ value.

The effects of temperature, pH, NaCl concentrations and the presence of divalent metal ions on the antimicrobial activity of LysSAP8 were assessed by a turbidity reduction assay [20, 37, 38]. To test the susceptibility of LysSAP8 at various factors, the endolysin was inoculated into the cell preparation (final conc. 1 μM), which was then incubated for 30 min with the following conditions: temperature (4°C, 15°C, 25°C, 30°C, 37°C, and 43°C), pH (50 mM citrate buffer pH 3–5, 50 mM imidazole-HCl buffer pH 6, and 50 mM tris-HCl buffer pH 7-9), NaCl (0, 500, 750, and 1,000 mM), and divalent metal ions (1 mM: Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, and Zn^{2+}, and EDTA for control). After incubation, the $O.D_{600}$ value was determined.

A viable cell counting assay was conducted to assess the lysis capability of LysSAP8 under the optimized condition. *S. aureus* KCCM 12103 was cultured to its exponential phase (>10^8 CFU/ml) and washed twice with the optimized buffer (Tris-HCl/500 mM, NaCl/1 mM $CaCl_2$, pH 8.0) by centrifugation (4,000 ×*g* for 10 min). LysSAP8 was inoculated into the washed cell suspension (final conc. 1 μM) and incubated at 30°C for 30 min. It was then diluted serially and spread on LB medium containing 1.5% agar. After overnight incubation at 37°C, individual colonies were counted.

Statistical Analysis

Statistically significant differences were evaluated using an unpaired one-tailed *t*-test for three independent experiments. *p*-values less than 0.01 were considered to be statistically significant.

Results and Discussion

Morphological Features of SAP8

The bacteriophage SAP8 infecting *S. aureus* was isolated from feces. The morphological analysis of SAP8 by transmission electron microscopy revealed that SAP8 has a long non-contractile tail approximately 300 nm in length, exhibiting typical characteristics representative of the *Siphoviridae* family with the adopted elongated head (Fig. 1).

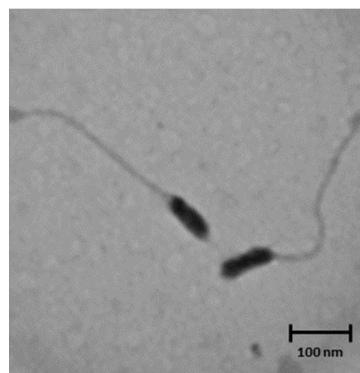

Fig. 1. Morphology of SAP8. A scale bar is presented at the bottom of the picture.

Table 1. Genomic features of the bacteriophage SAP8 infecting *S. aureus*.

Features	SAP8
Genomic size (bp)	45,533
No. ORFs	64
No. hypothetical genes	31
Encoding region (%)	92.77
G+C content (%)	33.41
No. tRNA	0

Genomic Features of SAP8

The genomic features of SAP8 were analyzed based on its complete sequence data (Table 1). The genome of SAP8 was 45,553 bp in size and was composed of 64 ORFs without tRNA. The G+C content of SAP8 was approximately 33%. The annotated genes from SAP8 were categorized into six functional groups and arranged (Fig. 2). Gene clustering in each functional group was found. Phage SAP8 harbored terminase and portal protein genes as essential packaging genes in many phages, promoting the translocation of viral genome into their capsid proteins. Also, phage SAP8 harbored eight structural genes, including head, tape measure protein, peptidase (tail lysin), and tail genes. In the DNA manipulation group, site-specific integrase present in SAP8 indicated that it possesses the ability to lysogenize host cells. For the host lysis, CHAP domain-containing protein as an endolysin of SAP8 (LysSAP8) and holin were located beside the site-specific integrase gene. In addition, a total of 13 genes for DNA regulation such as nuclease were encoded in the SAP8 genome.

Molecular Features of LysSAP8

The proteomic size and functional domains of LysSAP8 were predicted by bioinformatic analysis (Fig. 3A). The LysSAP8 proteome was determined to be 484 amino acids long and approximately 53 kDa in molecular weight. LysSAP8 was composed of an N-terminal CHAP, amidase-3 in the middle, and SH3b at the C-terminal end. CHAP and amidase-3, the proteins responsible for enzymatic

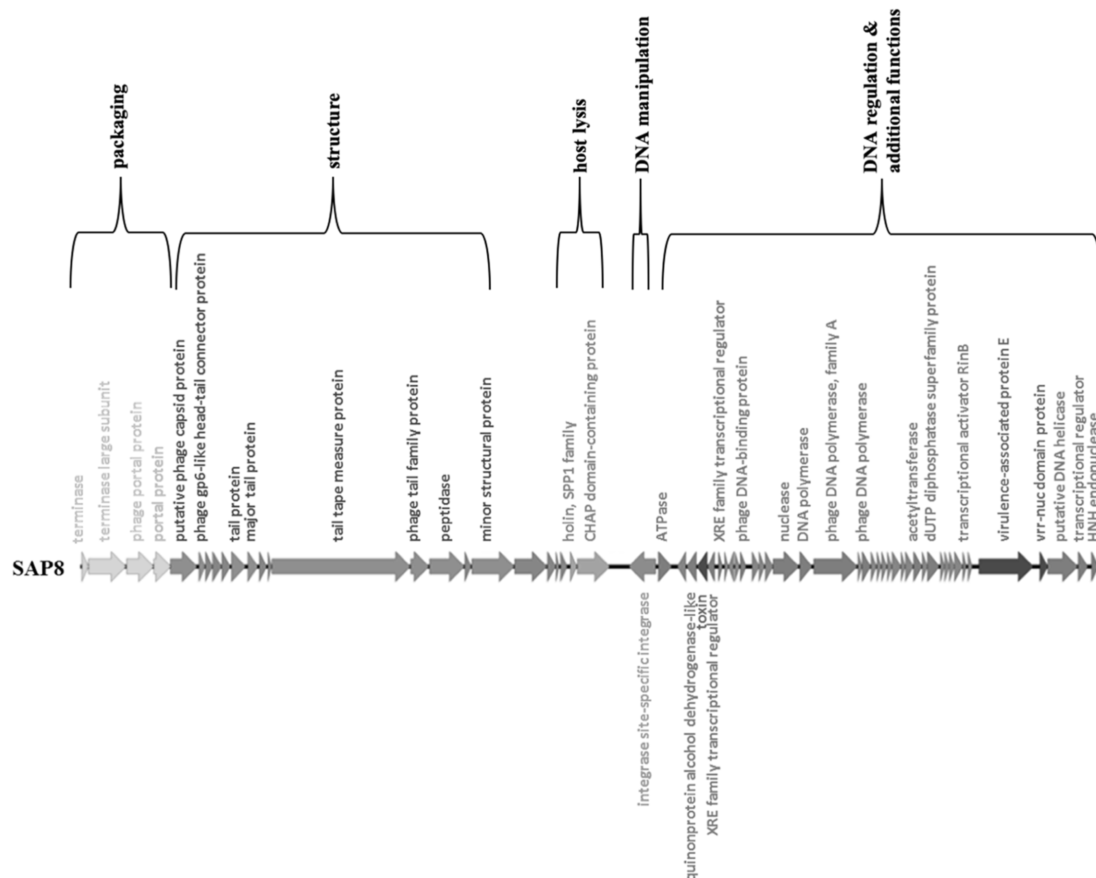

Fig. 2. Genetic organization of SAP8.

Genetic organization of SAP8. Predicted open reading frames are denoted by arrows.

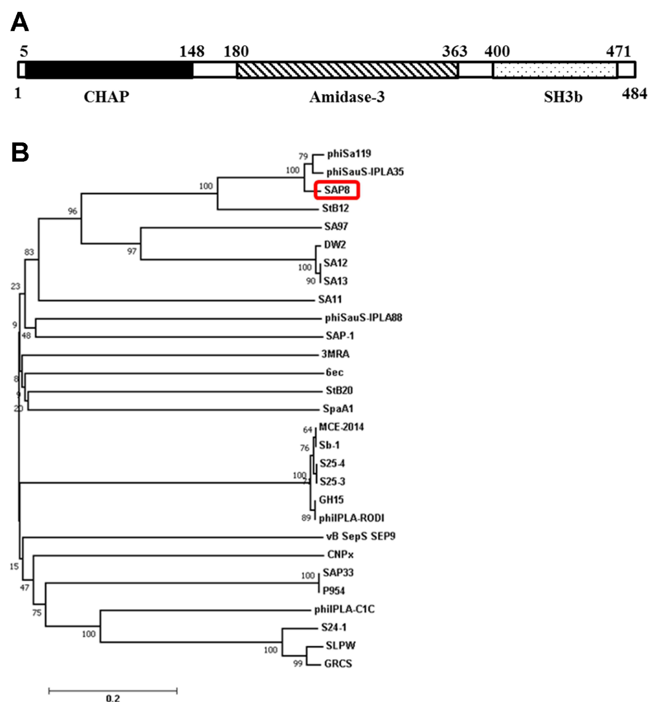

Fig. 3. (**A**) Modular organization of LysSAP8. (**B**) Phylogenetic tree for endolysins derived from bacteriophages infecting *S. aureus* including LysSAP8.

Black box: histidine-dependent amidohydrolase/peptidase (CHAP); Diagonal stripes: amidase-3; Black dots: SH3b. Unit: amino acid sequence.

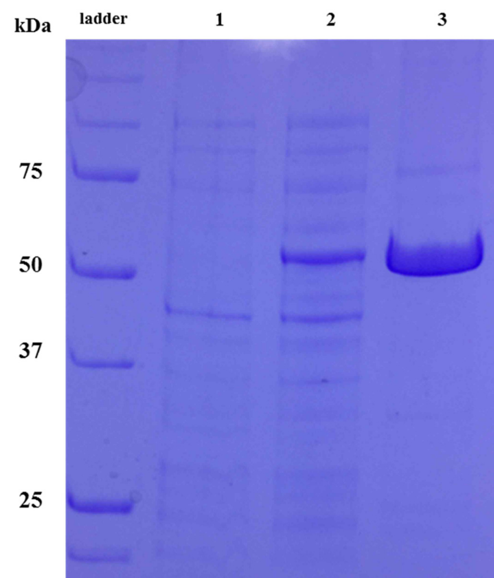

Fig. 4. SDS-PAGE analysis for expression and purification of LysSAP8 (55 kDa).

Lane 1: total lysate of IPTG-induced *E. coli* harboring the backbone plasmid; Lane 2: total lysate of IPTG-induced *E. coli* harboring the recombinant plasmid; Lane 3: partially purified endolysin.

degradation of peptidoglycan, were identified as enzymatically active domains (EADs) and SH3b was a typical cell wall-binding domain (CBD). Thus, it was determined that LysSAP8 was composed of two EADs and one CBD. These results showed that LysSAP8, as a LysK-like lysin, belongs to the type IV category, based on the grouping of staphylococcal endolysins [37]. Moreover, similar to LysK, LysSAP8 was determined to be approximately 53 kDa in molecular weight and composed of three domains [21, 22].

In the phylogenetic analysis, the taxonomical features of LysSAP8 were analyzed by comparing with those of other bacteriophage endolysins for *S. aureus* that were available in the NCBI database (Fig. 3B). The phylogenetic tree revealed that all endolysins analyzed here could be divided into two groups. Among each group, the characteristics of SAP8 were very similar to those of phiSA119 and phiSauS_IPLA35.

Host Spectrum, MBC, and Thermal Stability of LysSAP8

LysSAP8 overexpressed in *E. coli* was partially purified using nickel-chelating affinity chromatography. The molecular weight of LysSAP8 fused with histidine tag was found to be around 55 kDa. SDS-PAGE of the expressed bacterial fraction showed the presence of a dense protein band of the target size (Fig. 4).

Although phage SAP8 had relatively narrow host spectrum (15/75), LysSAP8 which was derived from phage SAP8 demonstrated lytic activity against a broad spectrum of the tested staphylococcal strains (Table S1). Among the 75 *S. aureus* isolates including MRSA, 73 strains showed susceptibilities to LysSAP8. Additionally, lysis activity of LysSAP8 against most of the other staphylococcal species (*S. epidermidis*, *S. hominis*, *S. sciuri*, *S. warneri*, *S. xylosus*) was also observed except for *S. condiment* PS10-3, *S. warneri* CCBK901, *S. epidermidis* KCCM 35494 and 40416. However, LysSAP8 killed neither the other gram-positive (*Bacillus*, *Enterococccus*, *Lactobacillus*, *Listeria*, *Streptococcus*) nor gram-negative (*Escherichia*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Vibrio*) strains. These results presented its advantages as a biocontrol agent that could be employed in the food industry and could enable targeting of specific pathogens without causing death of the commensal microflora. Interestingly, phage SAP8 could not infect any of the tested MRSA which were susceptible to LysSAP8. These results suggested that antibiotic resistant-bacteria might be susceptible to endolysin LysSAP8, even if they were

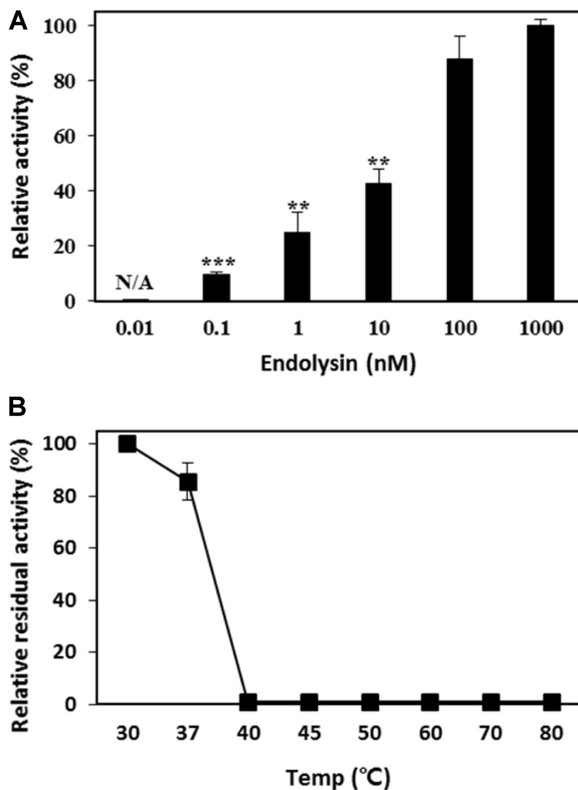

Fig. 5. Minimal inhibitory concentration (MIC) and protein thermal shift assays conducted on LysSAP8.
(**A**) Minimum concentration to kill the target bacteria, (**B**) Stability upon high temperature treatment for 30 min. The relative activities were calculated based on the highest turbidity reduction rate on each experiment. The data are presented as means of the standard deviations of the triplicate assays. $*p < 0.01$; $**p < 0.001$; $***p < 0.0001$; N/A: not available.

insensitive to phage SAP8 which was the source of LysSAP8.

To determine the MBC of LysSAP8, it was serially diluted (final conc. 1000, 100, 10, 1, 0.1, 0.01 nM) and inoculated with *S. aureus* KCCM 12103 as the target bacteria (Fig. 5A). LysSAP8 showed lytic activity depending on its concentration, and the highest activity that was not significantly different with those at 1,000 nM was observed at 100 nM. The lowest concentration at which LysSAP8 demonstrated bactericidal activity was 0.1 nM. This suggested that, from the viewpoint of cost efficiency, LysSAP8 might be the preferred bactericidal agent.

The stability of LysSAP8 under conditions of high temperature was determined (Fig. 5B). After heat treatment for 30 min, the residual enzymatic activity of LysSAP8 remained unchanged till 37°C. However, the residual enzymatic activity of LysSAP8 was lost with unrecoverable inactivation by following treatment above 40°C for 30 min.

These results showed a broad host spectrum of LysSAP8 against most of the *Staphylococcus* species tested in this study, suggesting their high-efficiency as biocontrol agents. Especially, a low MBC of LysSAP8 (0.1 nM ≒ 5.3 μg/ml) revealed a highly bactericidal effect against *S. aureus* in logarithmic growth phase, comparing those of endolysin LysK (1.4 μM) and P28 (125 μg/ml) [36, 39]. However, endolysin LysSAP8 had poor thermal stability as one of its drawbacks. Although LysSAP8 was less thermo-stable than other endolysins such as PlySs2 and LysSA11 [37, 40], several studies presented possible methods to improve thermal-stability of endolysin by using reverse micelles entrapping proteins or in combination with multiple thermo-stabilizing amino acid mutations [13, 41].

Effects of Temperature, pH, NaCl, and Divalent Metal Ions on Antibacterial Activity of LysSAP8

The antimicrobial activity of LysSAP8 was assessed using the turbidity reduction assay under the following conditions: temperature (4–43°C), pH (3–9), NaCl concentration (0–1,000 mM), and 1 mM concentration of various divalent metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, and Zn^{2+}).

It was found that LysSAP8 was completely active between temperatures of 15°C and 30°C (Fig. 6A). However, at temperatures below 4°C, the lytic activity was reduced to 58% and at temperatures over 37°C, nearly complete loss of its activity was observed. Interestingly, as shown in Fig. 5B, although the enzymatic activity of LysSAP8 was recoverable when it was pre-incubated at 37°C, the lysis activity of LysSAP8 was drastically low at this temperature.

On the buffers adjusted to pH values from 3 to 9, LysSAP8 demonstrated variations in lytic activity (Fig. 6B). The optimum pH of LysSAP8 was 7 to 8, but its activity was significantly decreased to less than 50% in the buffer adjusted to pH 6. Additionally, its lytic activity was almost lost in buffers at pH less than 5. Furthermore, at pH 9, the lytic activity of LysSAP8 was found to decrease.

In the experiment conducted to assess the effects of different concentrations of NaCl on the endolysin, lytic activity of LysSAP8 varied significantly with change in NaCl concentrations (Fig. 6C). In the absence of NaCl, the efficacy of LysSAP8 for lysis was approximately 50% of that in buffer supplemented with 500 mM NaCl, which was considered the optimum NaCl concentration. Moreover, its activity was found to decrease drastically in buffer containing 750 mM NaCl and was almost inactivated in 1,000 mM NaCl buffer.

The effects of divalent metal ions on the lysis activity were determined by testing LysSAP8 in the presence or

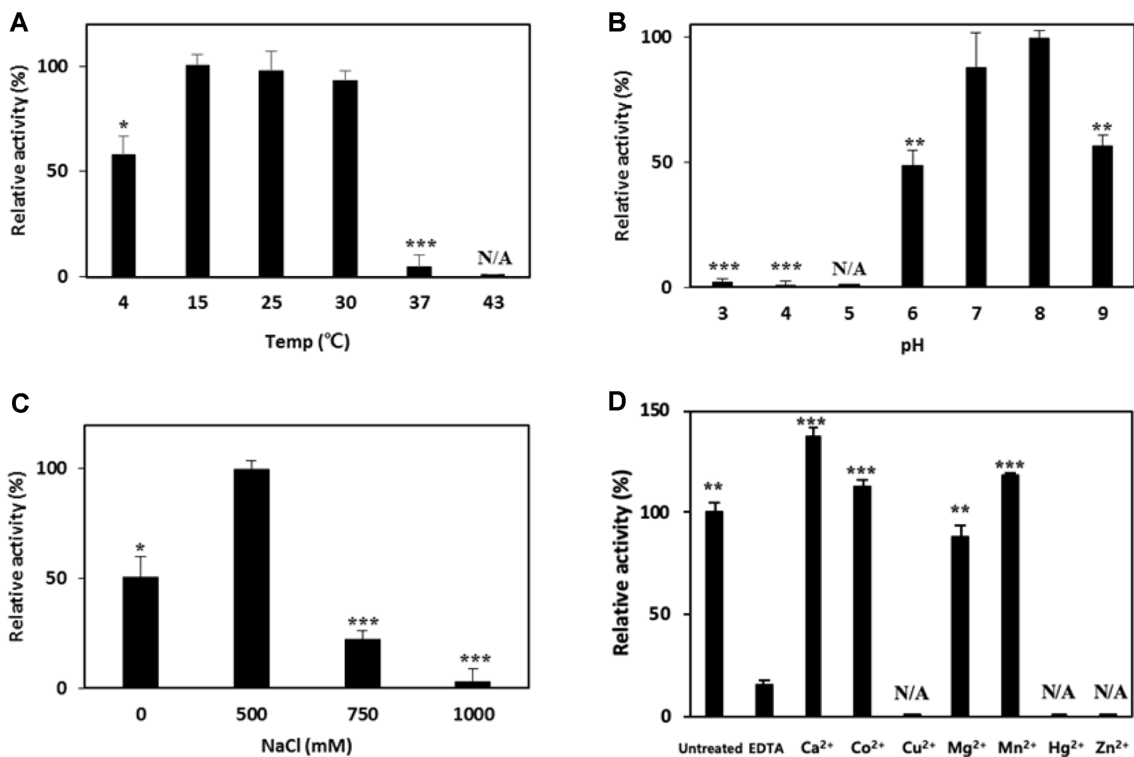

Fig. 6. Effects of temperature, pH, NaCl, and divalent metal ion on lytic activity of LysSAP8.
(**A**) Temperature (**B**) pH (**C**) NaCl (**D**) divalent metal ions. The relative activities of (**A**), (**B**), and (**C**) were calculated based on the highest turbidity reduction rate on each experiment, and the relative activities of (**D**) were calculated based on the turbidity reduction rate of the untreated control. The data are presented as means with standard deviations of triplicate assays. $*p < 0.01$; $**p < 0.001$; $***p < 0.0001$; N/A: not available.

absence of the ions (Fig. 6D). Lytic activity of LysSAP8 was found to decrease significantly upon treatment with EDTA suggesting that it might require metal ions for high efficacy of lysis activity. Furthermore, treatments with Cu^{2+}, Hg^{2+}, and Zn^{2+} ions inhibited lytic activity of LysSAP8. However, lytic activity of LysSAP8 treated with 1 mM of calcium ions was found to be approximately 138%, compared with the activity of the control. Furthermore, the activity of LysSAP8 was enhanced to 113% and 118% upon addition of solutions containing 1 mM Co^{2+} and Mn^{2+}, respectively.

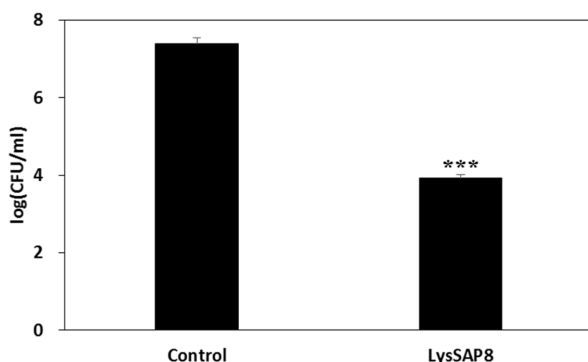

Fig. 7. Lysis capability of LysSAP8 under the optimized condition.
Lysis capability of LysSAP8 under the optimized condition. The data are presented as means of the standard deviations of triplicate assays. $***p < 0.0001$.

Consequently, its high efficacy of lysis can be achieved at 15–30°C in a buffer adjusted to pH 7–8 and supplemented with 500 Mm NaCl and calcium ions. These results revealed that LysSAP33 had similar or better biochemical characteristics comparing with other staphylococcal endolysins, and might be a good candidate for use as a biocontrol agent [20, 37]. Also, these optimized conditions suggest that the LysSAP8 might have good potential for applications in either food containing NaCl or neutral/sub-acid food.

Antimicrobial Activity of LysSAP8 under the Optimized Condition

Lytic activity of LysSAP8 under the optimized condition, as described in section 3.5, was examined by the viable cell

count (Fig. 7). When 1 μM of LysSAP8 was inoculated, high antimicrobial activity against the target cells in their exponential phase of growth was observed after 30 min, which resulted in a log reduction of approximately 3.46 log(CFU/ml). Compared to those of other staphylococcal endolysins, LysSAP8 demonstrated similar activity [19, 23]. However, LysSAP8 showed more rapid effects for reducing the target cells under the optimized condition. Especially, comparing with P-27/HP as one of the endolysins against *S. aureus*, LysSAP8 demonstrated tremendous capability as a biocontrol agent [42]. To reduce bacterial population, P-27/HP needs a minimum of 2 h, but LysSAP8 showed similar activity in 30 min using a half of the concentration of P-27/HP. These results suggested that LysSAP8 could be an alternative candidate for use as a potent biocontrol agent demonstrating its activity with rapid effect.

Acknowledgment

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Conflict of Interest

The authors have no financial conflict of interest to declare.

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