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Effective removal of staphylococcal biofilms on various food contact surfaces by *Staphylococcus aureus* phage endolysin LysCSA13

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ABSTRACT

Staphylococcal biofilms are recognized as a significant problem in the food industry because of their high resistance to antibiotics, and the use of bacteriophages or endolysins has been regarded as a promising alternative to antibiotics. In this study, LysCSA13, an endolysin from *S. aureus* virulent bacteriophage CSA13, was cloned and characterized. LysCSA13 consists of an enzymatic active domain and a cell wall binding domain. LysCSA13 showed strong antimicrobial activity against staphylococcal strains at pH 7.0–9.0, 4.0–37.0 °C and in the presence of Ca^{2+} and Mn^{2+}. In addition, a high efficacy of LysCSA13 in removing staphylococcal biofilms was observed on various surfaces, including polystyrene, glass and stainless steel, displaying an approximately 80–90% decrease in biofilm mass. Furthermore, 300 nM of LysCSA13 effectively removed staphylococcal sessile cells formed on stainless steel and glass by 1–3 log units compared with the untreated control. Scanning electron microscopy analysis visualized the effective deformation and removal of cells embedded in the biofilm matrix. The results indicate that LysCSA13 can effectively control staphylococcal planktonic cells and biofilms regardless of the contact surface matrix and suggest the possible use of LysCSA13 as a promising biocontrol agent in various food processing environments.

1. Introduction

Biofilm formation is one of the major concerns in the food industry, as the presence of biofilms can cause serious consequences to the human health due to the risk of developing foodborne diseases and outbreaks (Bridier et al., 2015; Gutierrez et al., 2016; Janssens et al., 2008). Staphylococci, including *Staphylococcus aureus,* are known to form biofilms on both biotic and abiotic surfaces and can persist in clinical and food settings, gaining high resistance to actions of typical antimicrobial agents, harsh environmental conditions, and host immunity (Li and Lee, 2017). Indeed, antibiotic therapies are generally unsuccessful for the treatment of biofilm-associated infections, mainly due to the low permeability of antibiotics to the biofilm matrix and the presence of a large number of persister cells with poor metabolic activity (Flemming and Wingender, 2010; Lewis, 2008; Stewart, 2002). Therefore, the development of novel *anti*-biofilm strategies has been strongly needed.

Bacteriophage-encoded endolysins have been the focus of research as an alternative to antimicrobials since they have a number of advantages over conventional antibiotics, such as potent species-specific activities and a low probability for developing bacterial resistance (Bai et al., 2016). Furthermore, endolysins can destabilize the biofilm structure by killing both replicating and nonreplicating bacteria embedded in the biofilm matrix, thereby disrupting the biofilm (Dunne et al., 2014; Sharma et al., 2018). The disruption of biofilms by endolysins has been reported in previous studies with *S. aureus* endolysin LysH5, LysK and phi11 endolysins (Fenton et al., 2013a; Gutierrez et al., 2014; Sass and Bierbaum, 2007a). The biofilm removal activity of an endolysin from the *Podoviridae S. aureus* phage was first studied with SAL-2. (Son et al., 2010b).

While these studies have focused on biofilms formed only on the surface of polystyrene, stainless steel and glass are also commonly used in the food processing environment. In addition to polystyrene, stainless steel and glass can be contaminated by food pathogens interacting with the surface, initiating cellular growth, and consequently leading to biofilm formation (Marques et al., 2007). This study proposed the use of an endolysin to reduce staphylococcal biofilms on various food contact surfaces, including polystyrene, stainless steel and glass.

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Table 1
The antimicrobial spectrum of LysCSA13.

Bacterial strain	Lytic activity (%)[a]	Reference or Source [b]
Staphylococcus strains		
Staphylococcus aureus ATCC 13301	+ + +	ATCC
MRSA CCARM 3089	+ + +	CCARM
MRSA CCARM 3090	+ + +	CCARM
MRSA CCARM 3793	+ +	CCARM
Staphylococcus aureus ATCC 6538	+ +	ATCC
Staphylococcus aureus ATCC 23235	+ +	ATCC
Staphylococcus aureus ATCC 29213	+ +	ATCC
Staphylococcus aureus Newman	+ +	Baba et al. (2008)
Staphylococcus aureus ATCC 33586	+ + +	ATCC
Staphylococcus aureus RN4220	+	Park et al. (2010)
Staphylococcus aureus ATCC 33593	+ + +	ATCC
Staphylococcus aureus ATCC 12600	+ + +	ATCC
Staphylococcus aureus laboratory isolate 62	+ + +	Korean sushi roll
Staphylococcus aureus laboratory isolate 63	+ + +	Korean sushi roll
Staphylococcus aureus laboratory isolate 130	+ + +	Animal
Staphylococcus aureus laboratory isolate 131	+ + +	Animal
Staphylococcus haemolyticus ATCC 29970	+ + +	ATCC
Staphylococcus hominis ATCC 37844	+ + +	ATCC
Staphylococcus warneri ATCC 10209	+ +	ATCC
Other Gram-positive bacteria		
Enterococcus faecalis ATCC 29212	–	ATCC
Bacillus cereus ATCC 14579	–	ATCC
Bacillus subtilis ATCC 23857	–	ATCC
Listeria monocytogenes ATCC 19114	–	ATCC
Gram-negative bacteria		
Salmonella enterica serovar Typhimurium SL1344	-[c]	ATCC
Escherichia coli MG1655 ATCC 47076	-[c]	ATCC
Cronobacter sakazakii ATCC 29544	-[c]	ATCC

[a] The percentage of lytic activity was obtained by the turbidity reduction assay for 60 min; 1–30% +, 30–70% + +; 71–100% + + +, 0% -.
[b] ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Culture; CCARM, Culture Collection of Antimicrobial Resistant Microbes.
[c] Gram-negative bacteria were treated with EDTA.

Previously, the virulent bacteriophage CSA13 infecting *S. aureus* was isolated and characterized for its morphology, physiology, and genomics (Cha et al., 2019). In this study, a putative endolysin gene, *lysCSA13,* was cloned and expressed in *Escherichia coli*, and the purified endolysin was biochemically characterized. The biofilm reduction efficacy of LysCSA13 was evaluated on various food contact surfaces to demonstrate its potential for use as a biocontrol agent.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

The bacterial strains used in this study are summarized in Table 1. All bacterial strains were grown in a tryptic soy broth (TSB) medium (Difco, Detroit, MI) at 37 °C with aeration. *E. coli* DH5α and BL21 (DE3) were grown in Luria-Bertani (LB) broth (Difco) at 37 °C and used in the cloning and expression of proteins, respectively.

2.2. Bioinformatics analysis

From the complete genome sequence of the *S. aureus* phage CSA13 (GenBank accession number MH107118), a gene (phCSA13_007) encoding the LysCSA13 endolysin was identified. The domain architecture of LysCSA13 was analyzed using the InterProScan program (https://www.ebi.ac.uk/interpro). The amino acid sequences of several staphylococcal endolysins harboring the CHAP domain and the SH3_5 domain were aligned using Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997) or Clustal X2.1 (Larkin et al., 2007).

2.3. Cloning and purification of LysCSA13

The endolysin gene (phCSA13_007) was PCR amplified using the following primers: LysCSA13_forward (5′-GCGGGATCCATGAAATCAC AAAAACAAGC-3′) and LysCSA13_reverse (5′-GCGAAGCTTTTATGAGA ACACCCCCCAAG-3′) (restriction sites are underlined). The resulting PCR product was subcloned into pET28a (Novagen, Madison, WI), which harbors an N-terminal hexahistidine (6 x His)-tag sequence. For LysCSA13 endolysin expression, *E. coli* BL21 (DE3) cells were transformed with the resulting plasmid. Protein expression was induced by the addition of 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG, final concentration) upon reaching an OD_{600} of 0.7, after which cells were further incubated at 18 °C for 20 h (Son et al., 2018). Cells were suspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0) and lysed by sonication (Branson Ultrasonics, Danbury, CT). The clarified supernatant containing soluble proteins was obtained by centrifugation at 15,000 × *g* for 30 min, followed by filtration (0.22-μm pore size; Millipore). Recombinant protein was purified using a Ni-nitrilotriacetic acid (NTA) Superflow column (Qiagen GmbH, Germany) according to the manufacturer's instructions. The identity and purity of the protein were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Dunne et al., 2014). Purified protein was exchanged into storage buffer (50 mM sodium phosphate, 300 mM NaCl, 30% glycerol, pH 8.0) using a PD Miditrap G-25 (GE Healthcare, Amersham, Bucks, UK) and stored at −80 °C until use.

2.4. Characterization of the LysCSA13 endolysin

The lytic activity of LysCSA13 was assessed by the turbidity reduction assay (Son et al., 2012). Briefly, exponentially growing cells (*S. aureus* RN4220) were harvested and suspended in reaction buffer (20 mM Tris-HCl, pH 8.0) to an OD_{600} of approximately 1.0 (Gaeng et al., 2000). After the addition of purified endolysin (30 nM–300 nM), the OD_{600} values were periodically monitored (0, 10, 20, 30, 40, 50, and 60 min). For Gram-negative bacteria, exponentially growing cells were pretreated with a buffer containing 20 mM Tris-HCl (pH 8.0) and 100 mM ethylenediaminetetraacetic acid (EDTA) for 5 min at 25 °C. The cells were washed three times with reaction buffer to remove residual EDTA prior to the addition of endolysin (Lim et al., 2012). To test the susceptibility of cells to LysCSA13 in various pH values, 300 nM of LysCSA13 was added to *S. aureus* RN4220 cell suspensions in the following buffers: 20 mM sodium acetate for pH 4.3–5.0; 20 mM Tris-HCl for pH 6.5–8.0; 20 mM glycine for pH 9.0; and 20 mM sodium carbonate for pH 10.0. To assess the effect of temperature on the enzymatic activity of LysCSA13, endolysins incubated at different temperatures (20–55 °C) were used in the turbidity reduction assay. To evaluate the thermal stability of the endolysin, the lysis assays were performed at 25 °C after the enzyme was incubated for 30 min at different temperatures. The influence of NaCl on the lytic activity was evaluated using different NaCl concentrations (0–100 mM) (Ha et al., 2018; Son et al., 2012). The effects of metal ions on the lytic activity were determined as previously reported (Schmelcher et al., 2012). To chelate metal ions attached to the endolysin, EDTA (5.0 mM; final concentration) was added to the endolysin (300 nM), and the mixture was incubated at 37 °C for 1 h. The EDTA was then removed by exchanging the endolysin into 20 mM Tris-HCl buffer (pH 8.0) using a PD trap G-25. The EDTA-treated enzyme was added to cell suspensions containing metal ions ($CaCl_2$, $MgCl_2$, $MnCl_2$, $CuCl_2$, or $ZnCl_2$; 1.0 mM final concentration), and the lysis activity was assayed in reaction buffer. The lytic activities were calculated after 1 h as follows: 100%{ΔOD_{600} test (endolysin added)-ΔOD_{600} control (buffer only)}/initial OD_{600}. The relative activities were calculated using the activity of enzyme which showed the

maximal activity in each condition.

2.5. Biofilm reduction assay on 96-well polystyrene plates

A biofilm disruption assay was performed as previously described with some modifications (Wu et al., 2003). Two different staphylococcal strains incubated in TSB medium supplemented with 0.25% D-glucose (Sigma-Aldrich, St. Louis, MO) were prepared and subcultured to the same media in a 96-well polystyrene microplate. After incubating the microplate for 24 h at 37 °C, all wells were washed with PBS. Once the biofilm was fixed (J Food Prot. 2005 May; 68(5):906-12., Crit Rev Microbiol. 2017 May; 43(3):313-351.), the experimental group wells were filled with LysCSA13 (50 nM–1000 nM), whereas reaction buffer (20 mM Tris-HCl, pH 8) was added as a negative control. After incubation for 2 h at 37 °C, each well was washed once with PBS and stained with 1.0% crystal violet. Additional washing with PBS, solubilizing with 33% acetic acid was performed. The absorbance of the obtained solution was measured at 570 nm, and the sessile biomass was presented as the A_{570} value. The relative A_{570} was calculated after 1 h as follows: A570 test (endolysin added or buffer only)/A570 control (buffer only).

2.6. Biofilm reduction assay against biofilm on stainless steel and glass surfaces

Stainless steel ($2 \times 2\,cm^2$, type: No. 4) coupons and glass cover slips ($2 \times 2\,cm^2$) were sterilized by dipping into 70% ethanol and autoclaving at 121 °C for 15 min. The biofilm formation and endolysin treatment were performed as described previously (Sillankorva et al., 2008), with some modifications. Briefly, stainless steel and glass coupons were placed on the wells of a 6-well microplate (Corning, New York, NY, USA), each well containing 3 ml of TSB medium supplemented with 0.25% D-glucose (Sigma). Bacterial culture was added to each well, and the microplate was incubated at 37 °C under static conditions for 24 h. Stainless steel coupons and glass coupons with biofilms were immersed in PBS twice and then placed in new microplates. After the fixation of the biofilm, the plates were incubated at 37 °C with different concentrations (100–1000 nM) of LysCSA13. Control experiments were performed under the same conditions with 3 ml of reaction buffer (20 mM Tris-HCl, pH 8). The staining and measurement of sessile biomass was performed as described above.

To determine the counts of bacteria attached to each coupon, a viable cell count assay was performed following a previously reported method with some modifications (Gutierrez et al., 2014). Once the biofilm was developed, the stainless steel and glass were swiped four times with a sterile swab and then immersed into 9 ml of PBS buffer. Vigorous shaking for 1 min allowed the disaggregation of the biofilm. Finally, several serial dilutions were plated onto tryptic soy agar (TSA) (Difco) and incubated at 37 °C.

2.7. Characterization of biofilm using field emission scanning electron microscopy (FESEM)

To prepare samples for scanning electron microscopy (SEM), the procedures of the biofilm assay on stainless steel coupons in 6-well culture plates were performed as described above. The microscopic sample was prepared following a previously reported method (Sillankorva et al., 2008). A field emission scanning electron microscope (Sigma 55VP, Carl Zeiss, United Kingdom) at the National Instrumentation Center for Environmental Management (NICEM, Seoul, South Korea) was used to observe the biofilm formation.

2.8. Statistical analysis

Statistical analysis was conducted using the GraphPad Prism program (version 5.01). The one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (95% confidence interval) was performed. The data are presented as means with standard deviations. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Identification and overexpression of LysCSA13 phage endolysin

From the genome sequence of phage CSA13, the predicted open reading frame for the endolysin gene was identified and this 747 bp-long ORF was designated as *lysCSA13*. According to the Pfam 28.0 analysis, LysCSA13 was predicted to be composed of the CHAP domain (PF05257, E-value, 2e-11) at its N-terminus and the SH3_5 domain (PF08460, E-value, 6.6e-23) at its C-terminus (Fig. S1A). BLASTP analysis showed that LysCSA13 had the highest homology to an amidase from *S. aureus* phage SAP-2 (SAL-2) (YP_001491539.1, 89% identity) (Son et al., 2010a) and that the endolysin of *S. aureus* phage phiP68 exhibited 85% homology with LysCSA13 (Fig. S1B) (Vybiral et al., 2003). To date, endolysins derived from *Podoviridae* family phages are very limited, and only endolysin SAL-2 has been studied for its removal activity of both planktonic cells and biofilms. Therefore, the study of LysCSA13, a novel endolysin from the *Podoviridae* family of bacteriophages, will be meaningful as a potential biocontrol agent.

LysCSA13 was cloned and overexpressed in *E.* coli. After purification, a total of 10 mg of LysCSA13 was obtained from 1 L of the expression culture and a single band of the purified endolysin was observed between 26 and 34 kDa by SDS-PAGE (Fig. S2). This result was consistent with the calculated molecular mass of LysCSA13 (28 kDa). The turbidity reduction assay revealed the effective lytic activity of LysCSA13 against *S. aureus* RN4220 in a concentration-dependent manner. After adding 300 nM (8.51 μg/ml) LysCSA13, the turbidity of the cell suspension (OD_{600}) dropped by 70% within 10 min. Treatment with 30 nM (0.85 μg/ml) LysCSA13 could exert nearly half of the maximal lytic activity (Fig. 1).

3.2. Biochemical characteristic of LysCSA13

LysCSA13 was highly active at pH 7.0–9.0, with the highest activity at pH 8.0 (Fig. 2A). The maximal activity of LysCSA13 was observed at 25 °C, and this endolysin could effectively lyse susceptible bacteria between 20 and 55 °C (Fig. 2B). LysCSA13 retained 80% and 20% of its lytic activity after heating 37 °C and 45 °C, respectively (Fig. S3). LysCSA13 exhibited a 50% reduction in lytic activity in the presence of

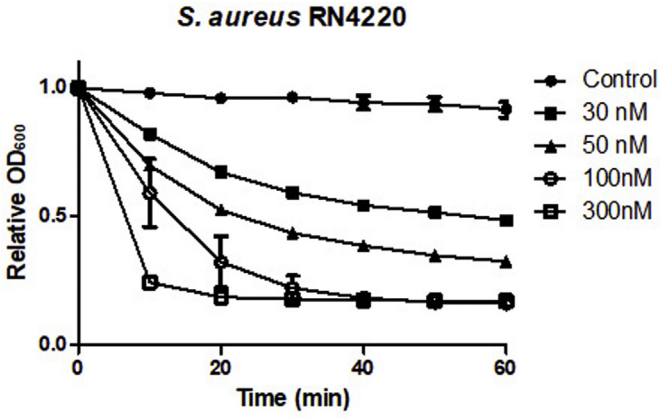

Fig. 1. Lysis of *S. aureus* RN4220 with four different concentrations of LysCSA13. Black circle negative control (no enzyme added), black square 30 nM (0.85 μg) enzyme added, black triangle 50 nM enzyme added, clear circle 100 nM enzyme added, clear square 300 nM enzyme added. The data shown are the mean values from three independent measurements, and the error bars represent the standard deviation.

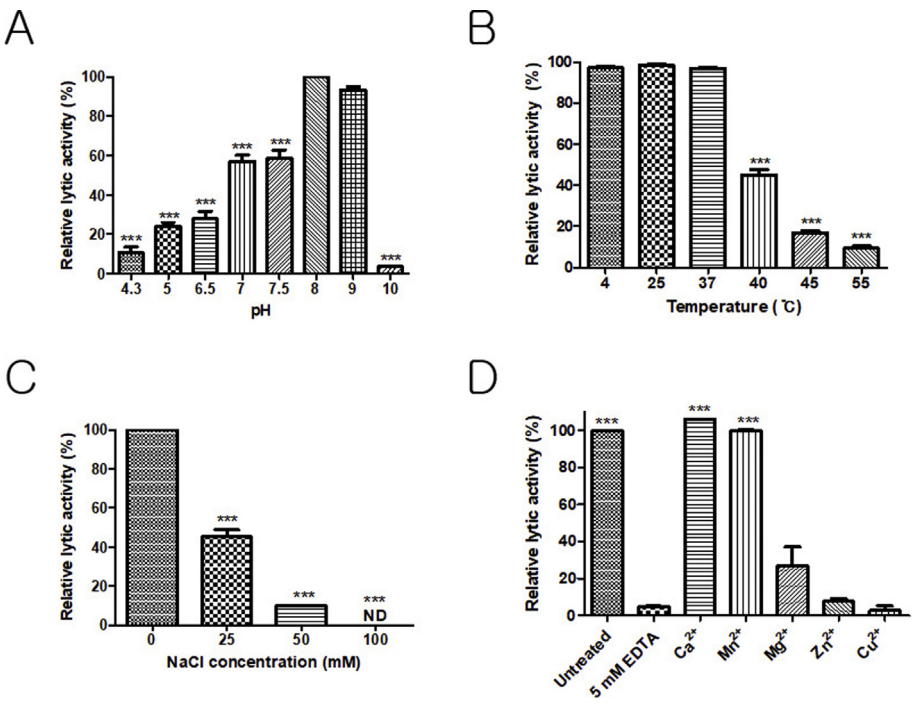

Fig. 2. Biochemical characterization of LysCSA13. The effects of pH (A), temperature (B), NaCl concentration (C) and metal ion (D) on the lytic activity of LysCSA13 against *S. aureus* RN4220 cells are shown. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. The asterisks indicate significant differences (***, $P < 0.0001$).

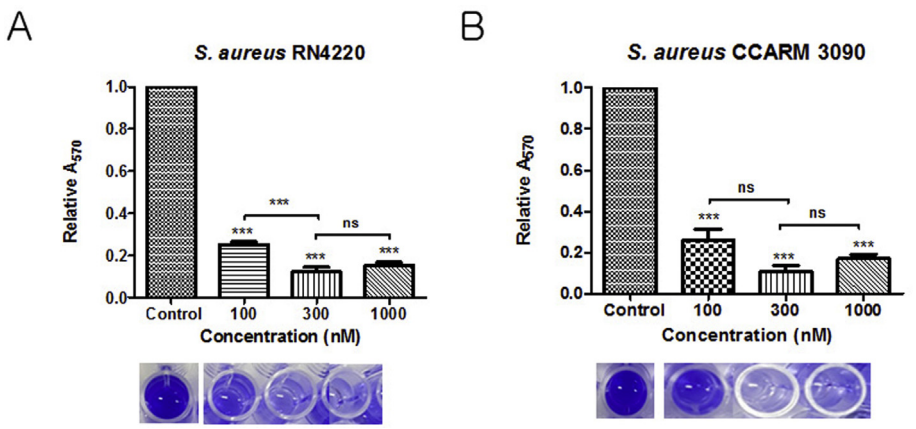

Fig. 3. Removal of 24 h-old biofilm on 96-well polystyrene microplate with LysCSA13. Biofilms of *S. aureus* RN4220 (A) and CCARM 3090 (B) were treated with three different concentrations of LysCSA13 for 2 h. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. The asterisks indicate significant differences as follows: ***, $P < 0.0001$; **, $P < 0.005$; *, $P < 0.05$; ns, not significant.

25 mM NaCl, indicating that this endolysin is sensitive to NaCl (Fig. 2C). To study the effects of divalent ions on the lytic activity of LysCSA13, the metal ions were removed from the protein using 5.0 mM EDTA. Treatment with 5.0 mM EDTA considerably decreased the lytic activity of LysCSA13, demonstrating the necessity of metal ions for its full lytic activity. When 1.0 mM Ca^{2+} or Mn^{2+} was added to the EDTA-treated endolysins, the lytic activity was fully restored. However, the addition of Mg^{2+}, Zn^{2+}, and Cu^{2+} resulted in little or no recovery of activity of the EDTA-treated endolysin (Fig. 2D). Taken together, LysCSA13 requires divalent metal ions, particularly Ca^{2+} or Mn^{2+}, for its enzymatic activity.

The antimicrobial spectrum of LysCSA13 was examined against 19 staphylococcal strains, and other Gram-positive and Gram-negative bacteria (Table 1). All tested *S. aureus* strains, including MRSA and 3 other staphylococcal strains, such as *S. haemolyticus, S. hominis* and *S. warneri,* were lysed by LysCSA13. On the other hand, this endolysin did not show lytic activity against other Gram-positive bacteria and Gram-negative bacteria. These results indicated that LysCSA13 has a limited antimicrobial spectrum in staphylococcal species, whose peptidoglycan is cross-linked via pentaglycine interpeptide bridges (Schleifer and Kandler, 1972).

3.3. Biofilm reduction efficacy of LysCSA13 on the polystyrene microplate

The efficacy of LysCSA13 in reducing staphylococcal biofilms was examined using a 96-well polystyrene microplate. Biofilm reduction efficacy was evaluated by the crystal violet staining method on two staphylococcal strains, including MRSA strains. LysCSA13 showed a similar extent of biofilm reduction efficacy for the tested strains and exhibited saturated activity at 300 nM (Fig. 3). These results suggest a high biofilm reduction efficacy of LysCSA13 towards staphylococcal biofilms formed on polystyrene surfaces.

3.4. Biofilm reduction efficacy of LysCSA13 on various contact surfaces

To investigate the biofilm reduction efficacy on various contact surfaces, the activity of LysCSA13 was additionally tested on stainless steel and glass (Miao et al., 2017a). Biofilms were formed using *S. aureus* RN4200 and *S. aureus* CCARM 3090 because LysCSA13 showed the highest biofilm reduction capacity for these strains on the polystyrene surface. When 300 nM or more of the endolysin was applied to the biofilms formed on stainless steel, 84% and 82% of the biofilm mass of *S. aureus* RN4200 and *S. aureus* CCARM 3090 were removed, respectively, compared to the untreated control (Fig. 4A and B). A more

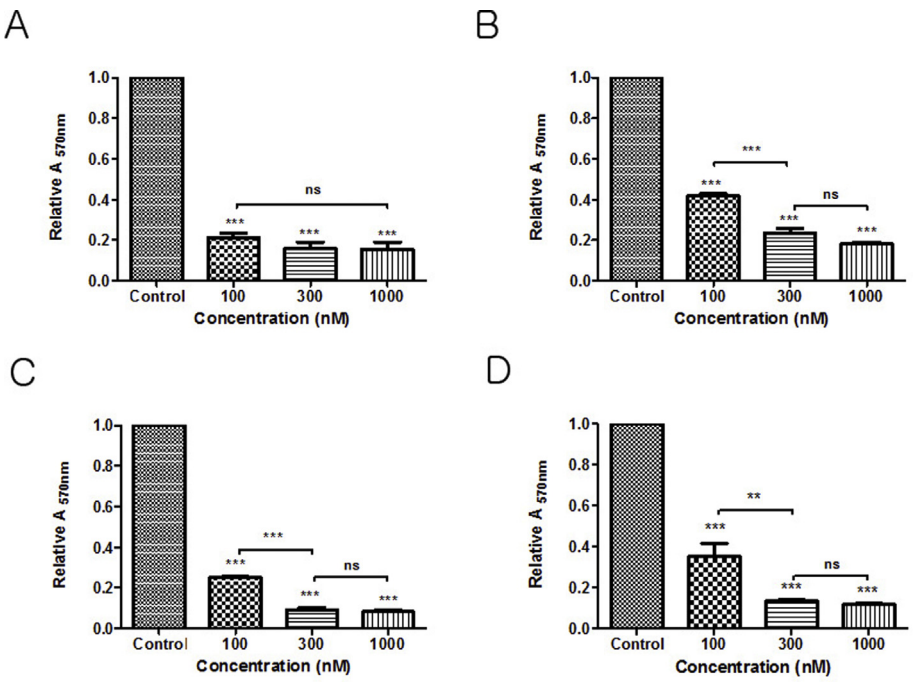

Fig. 4. Removal of 24 h-old biofilm on stainless steel and glass with LysCSA13. Biofilms of *S. aureus* RN4220 (A, C) and CCARM 3090 (B, D) were formed on stainless steel (A, B) and glass (C, D) treated with three different concentrations of LysCSA13 for 1 h. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. The asterisks indicate significant differences as follows: ***, $P < 0.0001$; **, $P < 0.005$; *, $P < 0.05$; ns, not significant.

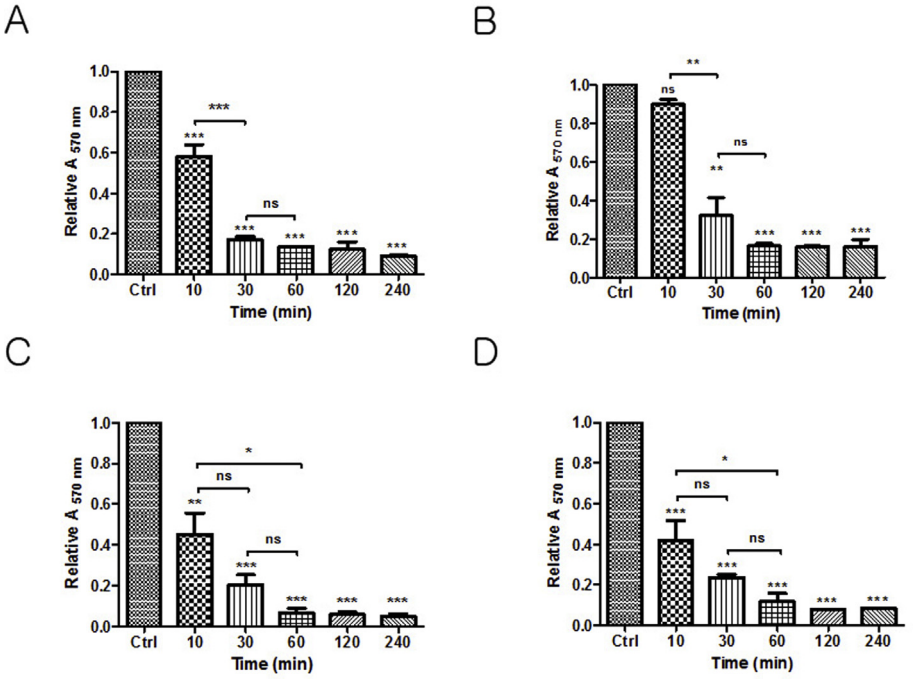

Fig. 5. Removal of 24 h-old biofilm for various treatment times with LysCSA13. Biofilms of *S. aureus* RN4220 (A, C) and CCARM 3090 (B, D) were formed on stainless steel (A, B) and glass (C, D) with five different treatment times. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. Asterisks indicate significant differences as follows: ***, $P < 0.0001$; **, $P < 0.005$; *, $P < 0.05$; ns, not significant.

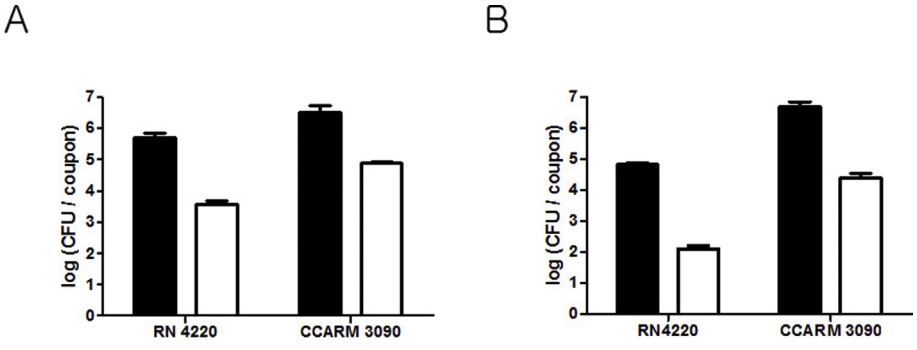

Fig. 6. Viable cell count of 24-h biofilm on various contact surfaces with LysCSA13. Twenty-four-hour biofilms of *S. aureus* RN4220 and CCARM 3090 were formed on stainless steel (A) and glass (B). Biofilms were treated with 300 nM of LysCSA13 (white) for 1 h for both strains. Control biofilms are represented in black. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation.

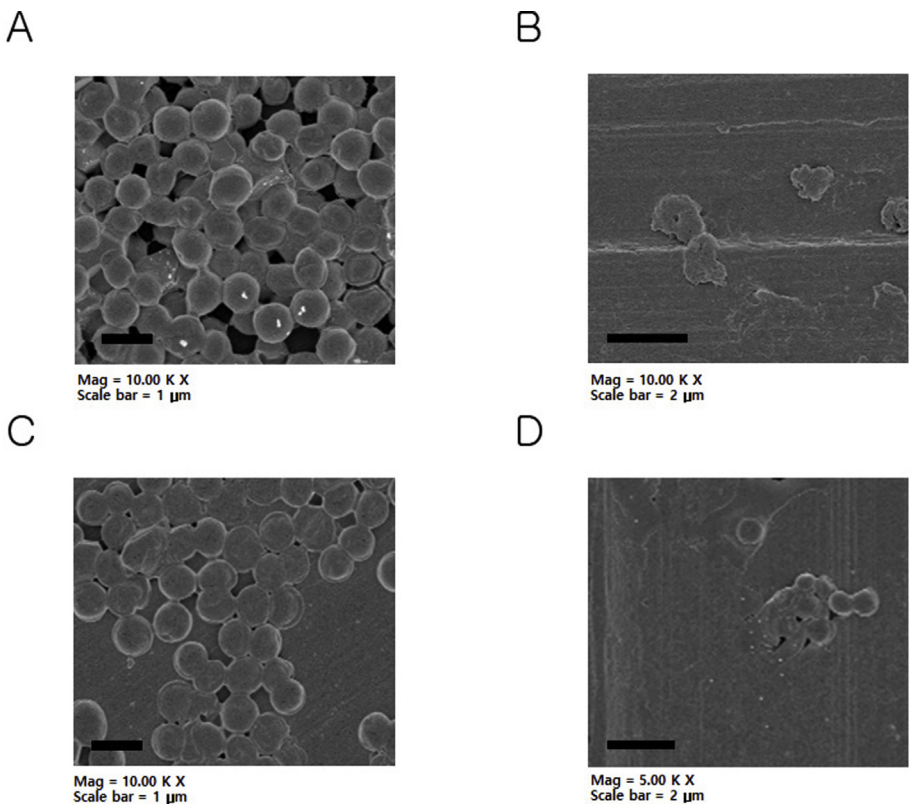

Fig. 7. FESEM analysis of the degradation efficacy of LysCSA13 against *S. aureus* RN4220 and CCARM 3090 on stainless steel. Biofilms formed by *S. aureus* RN4220 (A, B) and *S. aureus* CCARM 3090 (C, D) were treated with 300 nM of LysCSA13 for 4 h (*left*, before treatment; *right*, after treatment).

notable disruption of the biofilm was observed on glass than on stainless steel, removing 92% and 88% of the biofilm mass of *S. aureus* RN4200 and *S. aureus* CCARM 3090, respectively (Fig. 4C and D). When biofilms were treated for 5 different time periods, 30 min was sufficient to remove approximately 80% of the biofilm mass, and no further reduction of biofilm mass was observed after 1 h (Fig. 5). The results of a viable cell count assay show that LysCSA13 reduced the number of sessile cells by approximately 2 and 3 log orders of magnitude on stainless steel and glass, respectively (Fig. 6). These results indicate that LysCSA13 effectively reduces biofilms on various contact surfaces and that a 1 h treatment with 300 nM of LysCSA13 is sufficient for the removal of biofilms on both stainless steel and glass.

To strengthen these results, the biofilm reduction ability of LysCSA13 was further confirmed by FESEM analysis. After 24 h of incubation, biofilms of both *S. aureus* RN4220 and *S. aureus* CCARM 3090 were highly organized on stainless steel. The SEM images showed that *S. aureus* RN4220 produced more compact cell aggregates, forming a firm biofilm matrix, than *S. aureus* CCARM 3090 (Fig. 7A, C). When 300 nM LysCSA13 was treated for 4 h in biofilms, most cells were removed, and fewer cells remained on the stainless steel surface than on the control sample (Fig. 7B, D). Furthermore, the remaining cells appeared to be embedded in amorphous material, which could be the result of cell lysis (González et al., 2017). These results demonstrated that LysCSA13 has strong antibiofilm activity on stainless steel and glass as well as polystyrene surfaces.

4. Discussion

In this study, we identified a gene encoding the LysCSA13 endolysin from the *S. aureus*-specific bacteriophage CSA13 (Cha et al., 2019). LysCSA13 consists of an N-terminal CHAP domain and a C-terminal SH3 domain, and 11% of the reported *S. aureus* endolysins were involved in this domain composition (Chang and Ryu, 2017). The optimal lytic activity of LysCSA13 appeared at pH 8.0, in a wide range of temperatures and in the absence of NaCl. This enzyme required a divalent metal ion, such as Ca^{2+} or Mn^{2+}, for its full enzymatic activity. However, no Ca^{2+} or Mn^{2+} coordinating residues were detected through protein sequence analysis. In accordance with this finding, several CHAP domain-containing staphylococcal endolysins have been shown to require Ca^{2+} for their activity (Donovan et al., 2006; Gu et al., 2014; M. et al., 2011). Interestingly, no other endolysin targeting *S. aureus* studied beforehand showed a reactivation of enzymatic activity by Mn^{2+}. However, other endolysins, such as *Bacillus colistinus* endolysin CwlV (Shida et al., 2001), bacteriophage T5 endolysin (V. et al., 2009) and *Listeria* phage endolysins HPL118 and HLP511 (Schmelcher et al., 2012), showed effective recovery in enzymatic activity with Mn^{2+}.

LysCSA13 had antimicrobial activity against all 15 tested staphylococcal planktonic cells, including MRSA strains. Furthermore, LysCSA13 presented a notable disrupting activity against biofilms formed by *S. aureus,* including MRSA strains, showing a 70–90% reduction in OD_{570} after crystal violet staining compared to a LysCSA13-untreated control. LysCSA13 exhibited a maximum of 90% decrease in the *S. aureus* biofilm when 300 nM was added to the biofilm for 1 h, while other endolysins studied previously had lower biofilm removal efficacies. ClyF showed a reduction of 25–50% of biofilm mass when the biofilm was treated with 100 μg/ml of ClyF for 45 min (Yang et al., 2017). Another triple-acting fusion protein, a peptidoglycan hydrolase, presented an approximately 70% decrease in the *S. aureus* biofilm when the biofilm was treated with 1400 nM of the enzyme for 2 h (Becker et al., 2016). In the case of the CHAP domain of LysK, an approximately 60% decrease was detected when treated with 15.53 μg/ml of the enzyme for 4 h (Fenton et al., 2013b). In summary, LysCSA13 possesses high biofilm removal activity compared to other reported *S. aureus* endolysins.

The aim of this study was to remove biofilms on various food

contact surfaces using an endolysin. In this regard, we treated biofilms formed on stainless steel and glass with LysCSA13. Surfaces with high free energy, such as stainless steel and glass, are more hydrophilic and generally allow better attachment of bacteria, leading to biofilm formation at a higher rate in food processing environments (Chaturongkasumrit et al., 2011). LysCSA13 effectively reduced up to 93% of mature staphylococcal biofilm and a 3 log attached bacterial counts within 1 h. There was no significant difference in the biofilm reduction efficacy of LysCSA13 between stainless steel and glass in the crystal violet assay. However, a difference in the reduction of sessile cells adhered on the biofilms was observed between contact surfaces, as a 1 log-higher reduction of viable cell count was observed with biofilms formed on the glass surface. Generally, biofilms formed on stainless steel are more difficult to damage than glass since stainless steel has a rough surface, resulting in stronger cell adhesion (Miao et al., 2017b). This suggests that the effect of biofilm reduction by endolysins might differ depending on the contact surface.

Although several other *S. aureus* endolysins have recently been developed (Chang and Ryu, 2017; Sass and Bierbaum, 2007b; Son et al., 2010a), this study focused on the efficacy of one endolysin to remove staphylococcal biofilms encountered on various surfaces in food processing facilities. Our results revealed that LysCSA13 effectively eliminates not only staphylococcal planktonic cells but also biofilms, regardless of contact surfaces, including polystyrene, stainless steel and glass. Overall, this study demonstrates the potential use of LysCSA13 as an effective antibiofilm agent in various food processing procedures and environments.

5. Conclusion

This study demonstrated the removal of staphylococcal biofilms on various contact surfaces using LysCSA13, an endolysin from the *S. aureus* virulent phage CSA13. LysCSA13 showed strong lytic activity in a wide range of pH values and temperatures against all staphylococcal stains tested. In addition, LysCSA13 successfully removed more than 82%–92% of the biofilm mass on three contact surfaces, including polystyrene, stainless steel and glass. Considering its high staphylolytic activity and antibiofilm activity, endolysin LysCSA13 could be developed as a promising biocontrol agent in various food industries.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103245.

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