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Abstract

Streptococcus agalactiae is one of the pathogens in bovine mastitis, which contributes to substantial economic losses in the dairy industry. Endolysins are bacteriophage-encoded peptidoglycan degrading hydrolases, and they have become a novel weapon for selective elimination of target bacteria. This study aimed to identify endolysin with specific lytic activity against *S. agalactiae*, characterize the endolysin SALys78925, and evaluate its efficacy against related pathogens. The whole genome sequence of *S. agalactiae* strain was obtained from the National Center for Biotechnology Information database, and the putative gene for *SALys78925* was retrieved from their genomes. *SALys78925* was overexpressed in *Escherichia coli* BL21 (DE3) using pET system. The optimal conditions of SALys78925 were determined by assessing lytic activity under various ranges of pH, NaCl concentration, temperature, metal ions, and dosage. *In silico* analysis revealed that SALys78925 possesses a modular structure that comprises an N-terminal enzymatically active domain (Peptidase_M23) and a C-terminal cell wall binding domain (Zoocin A target recognition domain). SALys78925 exhibited maximal lytic activity against *S. agalactiae* KCCM 40417 at pH 9.0 and 37℃, but the activity decreased in a salt dose-dependent manner. The addition of Mn^{2+} enhanced SALys78925 activity, whereas Zn^{2+} reduced it by approximately 80%. Antimicrobial spectrum analysis revealed that SALys78925 exhibited varying degrees of lytic activity against multiple bacterial species, demonstrating potent lysis of *S. agalactiae* and lysis of *Streptococcus parauberis* and *Enterococcus faecalis*. These findings suggest that SALys78925 could be a promising candidate for preventing bovine mastitis in the dairy industry and beyond, offering a potential alternative to conventional antimicrobial treatments.

Keywords (3 to 6): endolysin, peptidoglycan hydrolase *Streptococcus agalactiae*, bovine mastitis, antimicrobial agent

INTRODUCTION

Bovine mastitis continues to cause decreased milk production, compromised milk quality, increased labor and treatment costs, and premature culling of infected animals, resulting in significant losses globally [1]. Bovine mastitis is an inflammatory disease that affects mammary glands and udder tissues. It can be caused by trauma, chemical irritation, or infections from various microorganisms such as fungi, viruses, algae, and especially bacteria [2]. Bovine mastitis is primarily caused by bacterial intra-mammary infection [3]. Bacterial infections are classified into two categories based on their source: contagious and environmental [4]. The primary etiological agents of contagious mastitis are bacteria that predominantly colonize the bovine mammary gland; they include *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*), and *Mycoplasma bovis* [5]. These pathogens can induce clinical, sub-clinical, or chronic mastitis, which may show symptoms of mammary inflammation and abnormal milk secretion, elevated somatic cell counts, or long-term udder damage that reduces milk production [6]. *S. agalactiae*, a gram-positive encapsulated coccus, is a facultative aerobe classified as group B *Streptococcus* (GBS) according to the Lancefield classification [7]. Historically, *S. agalactiae* was the predominant etiological agent of bovine mastitis [8]. The implementation and widespread adherence to the five-point hygienic plan since the 1960s have significantly reduced the incidence of *S. agalactiae*-associated mastitis in dairy herds [9]. Despite this decline, *S. agalactiae* remains a major mastitis pathogen in various regions worldwide. According to epidemiological studies conducted by Kabelitz et al., the geographical distribution of streptococcal mastitis reveals that *S. agalactiae* remains the primary causative agent in Africa and Asia, accounting for 49% and 40% of cases, respectively. Furthermore, in South America, *S. agalactiae* exhibits the highest prevalence among streptococcal species, representing 35% of mastitis cases [10]. Several countries like Denmark and Norway, which had previously controlled *S. agalactiae* infections, have reported the re-emergence of this pathogen [11, 12]. Additionally, *S. agalactiae* is associated with severe infections in humans, such as neonatal sepsis, endocarditis, meningitis, and pneumonia, which affect newborns, the elderly, and pregnant women [13-15]. Although antibiotics remain the primary treatment strategy, their effectiveness is increasingly limited, and the emergence of antibiotic-resistant pathogens poses a significant challenge to their continued use [16, 17]. Moreover, the report of *S. agalactiae* resistance to certain antibiotics has raised increasing concerns in public health [18], prompting the dairy industry to reduce the use of antimicrobial drugs [6].

Endolysins, also known as lysins or murein hydrolases, are hydrolytic enzymes produced by bacteriophages to degrade the cell wall of the host at the final stage of the lytic cycle [19]. Endolysins targeting gram-positive bacteria typically composed of combination of enzymatically active domain (EAD) and cell wall

binding domain (CBD) [20]. The CBD generally determines the lytic activity spectrum by binding to and recognizing the peptidoglycan (PG) layer [21], allowing endolysins to kill harmful bacteria without disrupting beneficial microbiota [19]. This characteristic makes them promising alternatives to antibiotics, with high specificity and low resistance rates. Over the past two decades, endolysins have demonstrated their efficacy in controlling bacterial contamination in the food industry and bacterial infections in the medical field [22, 23].

The traditional methodology for developing recombinant endolysins involves the isolation and screening of bacteriophages capable of lysing target bacteria, followed by the extraction of endolysin gene information from selected phages [24-26]. However, numerous studies have now employed computational design and bioinformatics tools to identify endolysin genes without phage isolation. Notably, the engineered NC5 endolysin exhibited pronounced synergistic effects with cloxacillin in the treatment of *S. uberis* mastitis [27]. Additionally, intracellularly active endolysins have been designed as fusion proteins, combining endolysin domains with cell-penetrating peptides or protein transduction domains to facilitate intracellular delivery [28]. Furthermore, the VersaTile technology enables the rapid assembly of engineered endolysins, which can subsequently undergo high-throughput screening under end-user conditions, such as raw milk. In contrast to these approaches, we identified a wild-type endolysin gene from *S. agalactiae* through comprehensive genomic analysis. This methodological approach aligns with recent innovations, such as the high-throughput genomic screening of methicillin-resistant *S. aureus* (MRSA), which identified 114 putative endolysin genes and led to the discovery of LyJH1892—a potent lytic endolysin with significant efficacy against MRSA [29].

Several endolysins have reported efficacy against *S. agalactiae*, including PlyGBS, LambdaSa1, LambdaSa2, B30, λSA2, PlySK1249, ClyR, EN534-C, λSa2lys, PlySs2, and PlySs9 [30-38]. These endolysins predominantly contain either CHAP or amidase as their EADs, coupled with SH3 as their CBDs. Therefore, the primary objective of this study was to develop a new endolysin that specifically targets *S. agalactiae*, composed of the Peptidase_M23 domain as the EAD and the ZoocinA_TRD as the CBD, based on genomic analysis. We sought to characterize the optimal lytic conditions and assess the lytic spectrum of the newly identified endolysin SALys78925. Our hypothesis is that the recombinant endolysin SALys78925, designed to target *S. agalactiae*, would exhibit specific bacteriolytic activity against the target pathogen.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All the bacterial strains used in the study are listed in Table 1. The recombinant endolysin SALys78925, which target *S. agalactiae*, was cloned in *E. coli* DH5α and expressed in *E. coli* BL21 (DE3). The *E. coli* strains DH5α (for cloning) and BL21 (DE3) (for expression) were cultured aerobically in Luria-Bertani (LB) broth (Difco Laboratories Inc., Detroit, MI, USA) at 37℃. The Korean Culture Center of Microorganisms (KCCM; Seoul, Republic of Korea) provided the type strain of *S. agalactiae* KCCM 40417 for the characterization of the SALys78925. For the lytic spectrum evaluation of SALys78925, species belonging to *Streptococcus*, *Staphylococcus*, and *Enterococcus* were obtained from KCCM, the National Culture Collection for Pathogens (NCCP; Cheongju, Republic of Korea), and the Korean Collection of Type Cultures (KCTC; Jeongeup, Republic of Korea). All the *Streptococcus*, *Staphylococcus*, and *Enterococcus* species used in this study were aerobically grown in Brain Heart Infusion (BHI) broth (Difco Laboratories Inc., Detroit, MI, USA) at 37℃.

Identification and cloning of putative endolysin gene (*SALys78925*)

SALys78925 was identified from the whole genome sequence of *S. agalactiae* strain deposited in GenBank (Accession Number: ALSX01000003.1) using the Rapid Annotations with Subsystems Technology (RAST) server [39]. Through lysis module analysis, a single candidate gene was selected and designated as SALys78925. The nucleotide sequence of *SALys78925* was subjected to codon optimization to enhance recombinant protein expression in *E. coli*. The optimized sequence, flanked by NdeI and XhoI restriction sites, was chemically synthesized and subsequently cloned into the pET-28b (+) expression vector with an N-terminal hexa-histidine tag (6xHis tag) sequence by Bionics Inc (BIONICS Co., Ltd., Seoul, Republic of Korea). The recombinant plasmid was transformed into $CaCl_2$-treated competent *E. coli* BL21 (DE3) cells via heat-shock transformation, according to the manufacturer's instructions (Enzynomics, Daejeon, Republic of Korea).

Expression and purification of recombinant endolysin, SALy78925

E. coli BL21 (DE3) transformed with *SALys78925* were cultured in LB broth containing kanamycin (50 μg/mL). The cultures were incubated until the optical density at 600 nm (OD_{600nm}) reached 0.4. Thereafter, recombinant protein expression was induced by the addition of 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG), with the concentration determined through empirical optimization. After induction, cultures were incubated at 16℃ for 18 h, and cells were harvested via centrifugation (10,000 g, 15 min, 4℃) and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Cell disruption was performed using a sonicator (KYY-80; Korea Process Technology Co., Ltd., Seoul, Korea) with four cycles of 1.5 min each under ice to prevent inactivation of recombinant proteins. The lysis buffer volume was reduced by half after each sonication cycle, and the supernatant was collected after each disruption step.

The recombinant protein SALys78925 was purified using Nuvia IMAC resin charged with Ni (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Proteins were eluted using a buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, and pH 8.0). The purified endolysin fractions were pooled and dialyzed in the same buffer without imidazole using an Amicon Ultra-4 (10 kDa MWCO) centrifugal filter device (Merck KGaA, Darmstadt, Germany) to facilitate the removal of residual imidazole and adjust the concentration to 1000 μg/mL. The molecular weight of the purified recombinant protein was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently tested for lytic activity against *S. agalactiae*. The final purified endolysin solution was stored at 4℃ for not more than two weeks.

Characterization of recombinant endolysin, SALys78925

The lytic activity of SALys78925 against *S. agalactiae* KCCM 40417 was assessed by monitoring the reduction in OD_{600nm} using an iMark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). Bacterial cultures were grown to mid-logarithmic phase (OD_{600nm} 0.8-1.0) at 37℃, harvested via centrifugation, and then washed with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and pH 8.0). The lytic activity of SALys78925 was determined by calculating the percentage reduction in OD_{600nm} over a 1 h incubation period. The reduction rate for both control (elution buffer) and SALys78925 treatment groups was calculated using the following formula:

$$\text{Reduction rate} = (OD_{inital} - OD_{1h}) / OD_{inital} \times 100$$

The lytic activity was expressed as the difference in reduction rate between the treatment and the control:

$$\Delta\text{Lytic activity} = (\text{Reduction rate}_{treatment} - \text{Reduction rate}_{control}).$$

All lytic activity measurements were performed in triplicate.

Each subsequent assay was conducted using the optimal conditions identified in preceding experiments. To determine the optimal pH, *S. agalactiae* cells were suspended in a series of buffers ranging from pH 6.0 to 10.0, each containing 50 mM sodium phosphate (pH 6.0 to 8.0) and 50 mM sodium glycine (pH 9.0 to 10.0). SALys78925 (final concentration: 100 μg/mL) was added to each suspension and incubated for 1 h at 37℃. The optimal temperature was determined by incubating SALys78925 (final concentration: 100 μg/mL) with *S. agalactiae* suspensions in the optimal pH buffer at 4, 16, 25, 37, 50, and 60℃ for 1 h. To examine the effect of NaCl concentration on the SALys78925 activity, we added various concentrations of NaCl (0, 62.5, 125, 250, 500, and 1,000 mM) to the optimal pH and measured the lytic activity of SALys78925 as described above for optimal temperature for 1 h. To assess the impact of divalent cations, SALys78925 was pretreated with 5 mM

ethylenediaminetetraacetic acid (EDTA) at 25℃ for 30 min to chelate divalent cations. The metal-depleted SALys78925 was subsequently incubated with 10 mM of either $CaCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$ at 25℃ for 30 min to facilitate cation repletion. The lytic activities of SALys78925 under these various metal ion conditions were then evaluated in the optimal buffer at the optimal temperature, as determined empirically. To conduct dose-response analysis, *S. agalactiae* suspensions in optimal buffer were treated with serially diluted SALys78925 (final concentration: 12.5 to 100 μg/mL) supplemented with optimal metal ions. The reactions were incubated at optimal temperature, and OD_{600nm} values were monitored at 5, 10, 15, 30, 45, and 60 min to track the kinetics of bacterial lysis.

Antimicrobial Spectrum of endolysin SALys78925

All *Streptococcus*, *Staphylococcus*, and *Enterococcus* species listed in Table 1 were cultured to mid-logarithmic phase (OD_{600nm} 0.8-1.0) as previously described, harvested via centrifugation, and then resuspended in the optimal buffer, which was determined empirically. The lytic activity of SALys78925 (final concentration: 100 μg/mL) against each bacterial strain was measured and calculated according to the method used for the characterization of SALys78925. Relative lytic activity (%) was calculated as the ratio of lytic activity against each tested bacterial strain to the lytic activity against *S. agalactiae* KCCM 40417.

Observation based on scanning electron microscopy (SEM)

The lytic activity of SALys78925 on *S. agalactiae* KCCM 40417 was visually assessed using the SEM as follows: *S. agalactiae* cells were harvested via centrifugation (7000 g, 15 min, 4℃) and resuspended in a 1×PBS solution. A 100 μL sample drop was deposited on microscope cover glasses (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Samples were fixed with 2.5% glutaraldehyde solution at 4℃ for 1 h, washed three times with 1×PBS solution and post-fixed with a 1% osmium tetroxide (OsO_4) at 4℃ for 1 h. Cells were subsequently dehydrated using an ascending ethanol series (50, 70, 95, and 100%), with 5-10 min exposure at each concentration. Samples were then dried at 37℃ and mounted on stubs. Prior to observation, samples were sputter-coated with a thin layer of platinum (Pt) (approximately 3 nm) using a Quorum G150TS sputter-coater (Lewes, UK). SEM analysis was performed using a Zeiss Gemini 500 SEM (Carl Zeiss AG, Oberkochen, Germany) at the Pusan National University Core Facilities in Pusan, Korea. Images were acquired at an accelerating voltage of 7.00 kV and a working distance of approximately 6.1 mm. High-resolution surface details were captured using an InLens detector at magnifications of 15,600× for the control sample and 11,900× for the treatment sample.

Statistical Analysis

The normality of all characterization data was assessed using the Shapiro-Wilk test in SAS 9.4 (SAS Institute Inc., NC, USA) package. The data for the pH and NaCl range test, which were confirmed to follow a normal distribution by the Shapiro-Wilk test, were analyzed using the PROC GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., NC, USA) according to the following model:

$Y_{ij} = \mu + T_j + E_{ij}$

Y_{ij} represents the response variable, μ is the overall mean, T_j represents the fixed effect of treatment (j = 1 to n), where n indicates the number of treatment groups in the dataset, and E_{ij} is the residual error. Differences among treatments were compared with the Tukey's range test if a significant overall treatment effect was observed.

The data for the temperature and metal test, which were determined to deviate from a normal distribution, were analyzed using the non-parametric Kruskal-Wallis test in R software (R version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria). For variables showing a significant effect, Dunn's multiple comparison test was performed using the dunnTest function from the FSA package, with p-values adjusted using the Benjamini-Hochberg false discovery rate method [40]. Statistical significance was declared at $p < 0.05$ and statistical trend was considered at $0.05 \leq p < 0.15$.

RESULTS

Sequence analysis of *SALys78925*

In silico analysis of *SALys78925* using the InterPro database revealed a modular structure with two distinct domains (Fig. 1). The N-terminal domain was identified as a Peptidase_M23 domain (Pfam: PF01551, InterPro: IPR016047, *e*-value = 3.0×10^{-25}), which is associated with a hydrolytic activity. The C-terminal domain was identified as the Zoocin A target recognition domain (Zoocin A_TRD) (Pfam: PF16775, InterPro: IPR031898, *e*-value = 1.6×10^{-46}), which plays a role in recognizing and binding to the PG layer of the target bacteria. Based on the primary structure, SALys78925 comprised 300 amino acid residues, and the calculated isoelectric point of SALys78925 was 9.24. The instability index was calculated to be 16.97, which is significantly below the value of 40, indicating that SALys78925 is likely to be a stable form of the protein [41].

Overexpression and purification of SALys78925

The expression of SALys78925 in *E. coli* BL21 was optimized using 0.5 mM IPTG induction for 18 h at 16℃ in LB broth containing kanamycin (50 μg/mL). SDS-PAGE analysis revealed a major band that corresponded to the purified soluble endolysin SALys78925 at approximately 32 kDa (Fig. 2). SALys78925 expression after induction and purification produced approximately 3.15 mg per liter of *E. coli* expression culture.

Characterization of SALys78925

S. agalactiae KCCM 40417 was selected as the type strain to measure the lytic activity of SALys78925. The highest lytic activity of SALys78925 against *S. agalactiae* was at pH 9.0 (Fig. 3A). The NaCl concentration effect test showed that the lytic activity of SALys78925 decreased in a salt dose-dependent manner (Fig. 3B). Thus, the optimal temperature was evaluated at pH 9.0 in the absence of NaCl, as previously identified. The temperature at which the highest lytic activity of SALys78925 was observed was at 37℃ (maintained between 16-37℃) (Fig. 3C). Following this, the metal ion test was evaluated at pH 9.0, 37℃, and in the absence of NaCl. SALys78925 exhibited the highest lytic activity against *S. agalactiae* when 10 mM Mn^{2+} was added to EDTA-treated SALys78925; however, the addition of Zn^{2+} reduced the activity of SALys78925 by ~80% (Fig. 3D).

Optimal lytic activity and observation based on SEM

A dose-response test and SEM analysis of SALys78925 against *S. agalactiae* KCCM 40417 was performed under optimal conditions (pH 9.0, 37℃, no addition of NaCl, and addition of 10 mM Mn^{2+}).

SALys78925 reduced the optical density of *S. agalactiae* in a dose-dependent manner above a concentration of 12.5 μg/mL, and a concentration of 50-100 μg/mL inhibited nearly 80% of *S. agalactiae* in 30 min of incubation (Fig. 4A). As shown in Fig. 4B, the lytic activity of SALys78925 against *S. agalactiae* could be partially attributed to the cell membrane integrity disruption. Moreover, SEM revealed that *S. agalactiae* cells exposed to SALys78925 had a deformed configuration compared to the control sample.

Antimicrobial spectrum of SALys78925

To evaluate the lytic spectrum of SALys78925, we further analyzed its lytic activity under optimal conditions against seven *Streptococcus* species (three *S. agalactiae* strains, *S. dysgalactiae*, *S. uberis*, *S. iniae*, and *S. parauberis*), four species of coagulase-negative staphylococci (CNS): *S. epidermidis*, *S. haemolyticus*, *S. simulans*, and *S. chromogenes*, two *S. aureus* strains, and *E. faecalis* (Table 1).

SALys78925 exhibited varying degrees of lytic activity against multiple bacterial strains (Fig 5). SALys78925 showed high lytic activity (relative lytic activity > 80%) against *S. agalactiae* strains (KCCM 40417, NCCP 14729, and KVCC-BA2100490) and moderate lytic activity (~ 60%) against *S. parauberis* KCCM 43262 and *E. faecalis* KCTC 5191. In contrast, SALys78925 showed low lytic activity (approximately 20%) against *S. uberis* KCTC 21183. However, SALys78925 exhibited extremely low or negligible activity against *S. dysgalactiae* KVCC-BA2100518, *S. iniae* KCTC 3657 and all staphylococcal species tested.

DISCUSSION

Bovine mastitis is a prevalent and costly infectious disease in the dairy industry, negatively affecting production efficiency, milk quality, and animal welfare. *S. agalactiae* is considered as a major pathogen for mastitis [5]. In the this study, we identified endolysin from *S. agalactiae* genome information using bioinformatical approaches and developed new endolysin with lytic activity against *S. agalactiae.*

As mentioned in the introduction, endolysins targeting gram-positive bacteria typically comprise two conserved domains: at least one EAD and one CBD [20]. Analysis using the InterPro database revealed that SALys78925 possesses an N-terminal EAD belonging to the Peptidase_M23 domain and a C-terminal CBD of the ZoocinA_TRD (Fig. 1). The Peptidase_M23 domain is a conserved catalytic domain present in various bacterial peptidoglycan hydrolases. Enzymes with this domain play crucial roles in bacterial physiology by cleaving specific bonds within the peptidoglycan layer of bacterial cell walls [42-44]. The Peptidase_M23 domain of Zoocin A and EnpA cleaves D-alanine–L-alanine bonds in the peptidoglycan cross-links of *Streptococcus* and *Enterococcus* species [45, 46]. Thus, the EAD of SALys78925, which belongs to the M23

peptidase family, likely plays a key role in bacterial cell wall degradation during the lytic process. Several endolysins containing an Peptidase_M23 domain have demonstrated potent lytic activity against *Streptococcus* species, including *S. pneumoniae*, *S. pyogenes*, *S. zooepidemicus*, and *S. oralis* [47-50]. The CBD primarily functions to enhance the enzymatic activity of the EAD by recognizing and binding specific ligands within the PG layer of target bacteria. The ZoocinA_TRD superfamily represents the CBD of Zoocin A, an exoenzyme secreted by *Streptococcus equi* subspecies *zooepidemicus* 4881 [51]. Zoocin A has demonstrated lytic activity against several streptococcal species associated with streptococcal pharyngitis and dental caries [52]. Additionally, a previous study reported that the endolysin LyJH307, which possesses the Zoocin A_TRD as its CBD, demonstrates potent lytic activity against *Streptococcus bovis* and is effective against several streptococcal species isolated from the rumen [53]. Based on this information, we employed turbidity reduction assays to evaluate the lytic spectrum of SALys78925 against various streptococcal species. SALys78925 exhibited potent lytic activity against *S. agalactiae* and a narrow lytic spectrum among the tested species. SALys78925 (100 μg/mL) effectively lysed *S. parauberis* and *E. faecalis* (relative lytic activity > 55%). Although *Enterococcus* and *Streptococcus* are now classified as separate genera, they were previously grouped together due to shared morphological and biochemical characteristics [54]. The ability of Zoocin A to bind specifically to *E. faecalis* likely results from these similarities. However, SALys78925 exhibited low or negligible activity against other staphylococcal species tested. The lytic spectra of endolysins targeting *S. agalactiae* exhibited notable variations across different studies. Endolysin PlyGBS showed efficacy against multiple strains of GBS [35]. In contrast, B30 demonstrated a broader lytic activity against *Streptococcus* groups A, B, C, E, and G [55]. λSa2 exhibited lytic activity against a range of species such as *S. agalactiae*, *S. dysgalactiae*, and *S. uberis* [30]. The EN572-5 showed lytic activity against several *Streptococcus sp*ecies, with the exceptions of *S. mutans* and *S. thermophilus* [56]. The differences in these lytic spectra likely stem from the domains that comprise each endolysin. SALys78925 represents a unique composition: a Peptidase_M23 domain and a ZoocinA_TRD domain. This unique domain composition may account for its specific lytic profile, differentiating it from previously characterized endolysins targeting *S. agalactiae*.

The endolysin SALys78925 demonstrated optimal lytic activity at pH 9.0, which differs from several reported for other *S. agalactiae*-targeting endolysins such as PlySK1249 (pH 7.0-8.5) and EN572-5 (pH 5.0-8.0) [32, 56]. The elevated pH optimum of SALys78925 correlates with its predicted pI of 9.24, suggesting that the protein's net charge properties may facilitate enhanced catalytic activity under alkaline conditions. The normal body temperature of dairy cows typically ranges from 33℃ to 36℃, with temperatures rising above 37℃ under various health disorders, including mastitis [57]. Moreover, 37℃ is the optimal growth temperature for most mastitis-causing microorganisms, including common pathogens such as *S. aureus* and *S. agalactiae*. The

endolysin SALys78925 exhibited maximal lytic activity at 37℃, but showed potential activity across a temperature range of 16 to 37℃. This suggests that SALys78925 would be effective within the physiological temperature range of dairy cows, both in healthy and disease states. Divalent metal ions are known to influence endolysin activity by binding to amino acid residues in their domains [53, 58]. For instance, the streptococcal endolysin LyJH307 demonstrated calcium-dependent lysis against *Streptococcus bovis* group [53]. Similarly, Ply700 exhibited calcium-dependent lysis against *S. uberis*, *Streptococcus pyogenes*, and *S. dysgalactiae*, while showing little activity against *S. agalactiae*, *S. aureus*, or *E. coli* [59]. In the present study, the activity of SALys78925 against *S. agalactiae* was enhanced in the presence of Mn^{2+}. However, there is no significant difference between the activity of Mn^{2+}-treated and non-treated SALys78925 ($p < 0.05$). This finding suggests that SALys78925 retains its efficacy independent of Mn^{2+} supplementation. Consequently, SALys78925 could be utilized effectively without the need for pretreatment or supplementation with divalent metal ions, demonstrating its potential application in therapeutic or prophylactic contexts. Under optimal conditions, SALys78925 (50-100 μg/mL) not only disrupted *S. agalactiae* cells as observed via SEM but also reduced viable bacterial cell counts within 5 min (Fig. 4). These findings revealed the potential of SALys78925 as a therapeutic agent against *S. agalactiae* infections. However, its therapeutic potential as a biocontrol agent for bovine mastitis requires further validation. While *in vitro* studies have demonstrated the promising bacteriolytic activity of endolysins against major mastitis-causing pathogens, clinical investigations assessing their therapeutic efficacy in bovine mastitis remain scarce. Comprehensive clinical trials are essential to establish the safety profiles of endolysins for therapeutic applications in bovine mastitis. Recent studies have stressed the importance of evaluating endolysin efficacy in raw cow's milk, given that various biological components in milk can affect its stability and bactericidal activity [28]. Furthermore, the cytotoxicity and oxidative response of PlyC endolysin were investigated in bovine neutrophils, demonstrating that it was non-toxic and did not alter immune cell function [60]. These findings highlight the need for subclinical trials to evaluate endolysin activity under conditions that closely mimic the natural mammary environment before advancing to large-scale clinical applications.

Despite these challenges, SALys78925 demonstrates considerable promise, as its specific lytic activity against *S. agalactiae*, *S. parauberis*, and *E. faecalis* provides an opportunity to establish a comprehensive antimicrobial profile against these key mastitis-causing pathogens. This could offer valuable insights for the development of targeted therapeutic strategies in the future.

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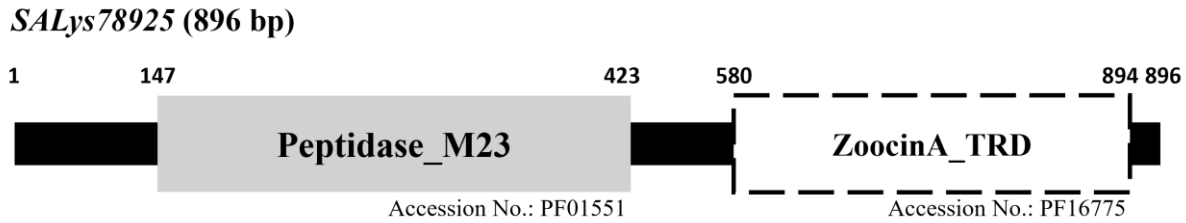
Tables and Figures

Table 1. Bacterial strains and growth conditions

Bacterial Strain	Purpose	Growth Media[1]
Escherichia coli DH5α	Cloning host	LB broth
Escherichia coli BL21 (DE3)	Expression host	LB broth
Streptococcus agalactiae KCCM 40417	Characterization	BHI broth
Streptococcus agalactiae NCCP 14729	Antimicrobial spectrum	BHI broth
Streptococcus agalactiae KVCC-BA2100490	Antimicrobial spectrum	BHI broth
Streptococcus dysgalactiae KVCC-BA2100518	Antimicrobial spectrum	BHI broth
Streptococcus uberis KCTC 21183	Antimicrobial spectrum	BHI broth
Streptococcus iniae KCTC 3657	Antimicrobial spectrum	BHI broth
Streptococcus parauberis KCCM 43262	Antimicrobial spectrum	BHI broth
Staphylococcus aureus NCCP 14754	Antimicrobial spectrum	BHI broth
Staphylococcus aureus NCCP 16830	Antimicrobial spectrum	BHI broth
Staphylococcus chromogenes KCTC 3579	Antimicrobial spectrum	BHI broth
Staphylococcus epidermidis KVCC-BA0001452	Antimicrobial spectrum	BHI broth
Staphylococcus haemolyticus NCCP 14693	Antimicrobial spectrum	BHI broth
Staphylococcus simulans NCCP 16236	Antimicrobial spectrum	BHI broth
Enterococcus faecalis KCTC 5191	Antimicrobial spectrum	BHI broth

[1]LB broth = Luria Bertani broth; BHI broth = Brain heart infusion broth



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506 **Fig. 1. Conserved domain structure of endolysin SALys78925.** The gray square indicates the enzymatically
507 active domain (Peptidase_M23), whereas the white square represents the cell wall binding domain (Zoocin
508 A_TRD).

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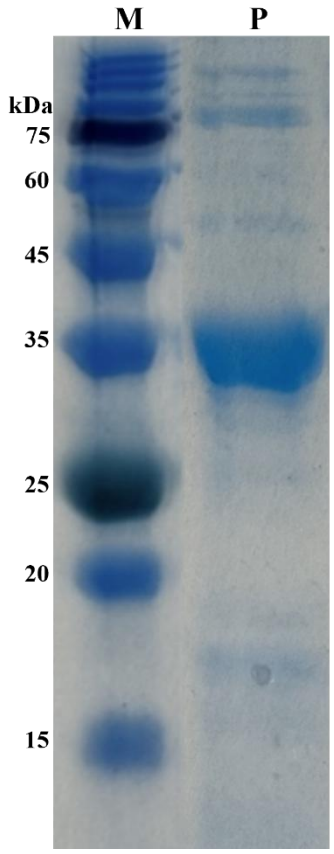


Fig.2. SDS-PAGE analysis for expression and purification of SALys78925. M, stained protein molecular weight markers: the marker sizes are denoted on the left; p, purified SALys78925

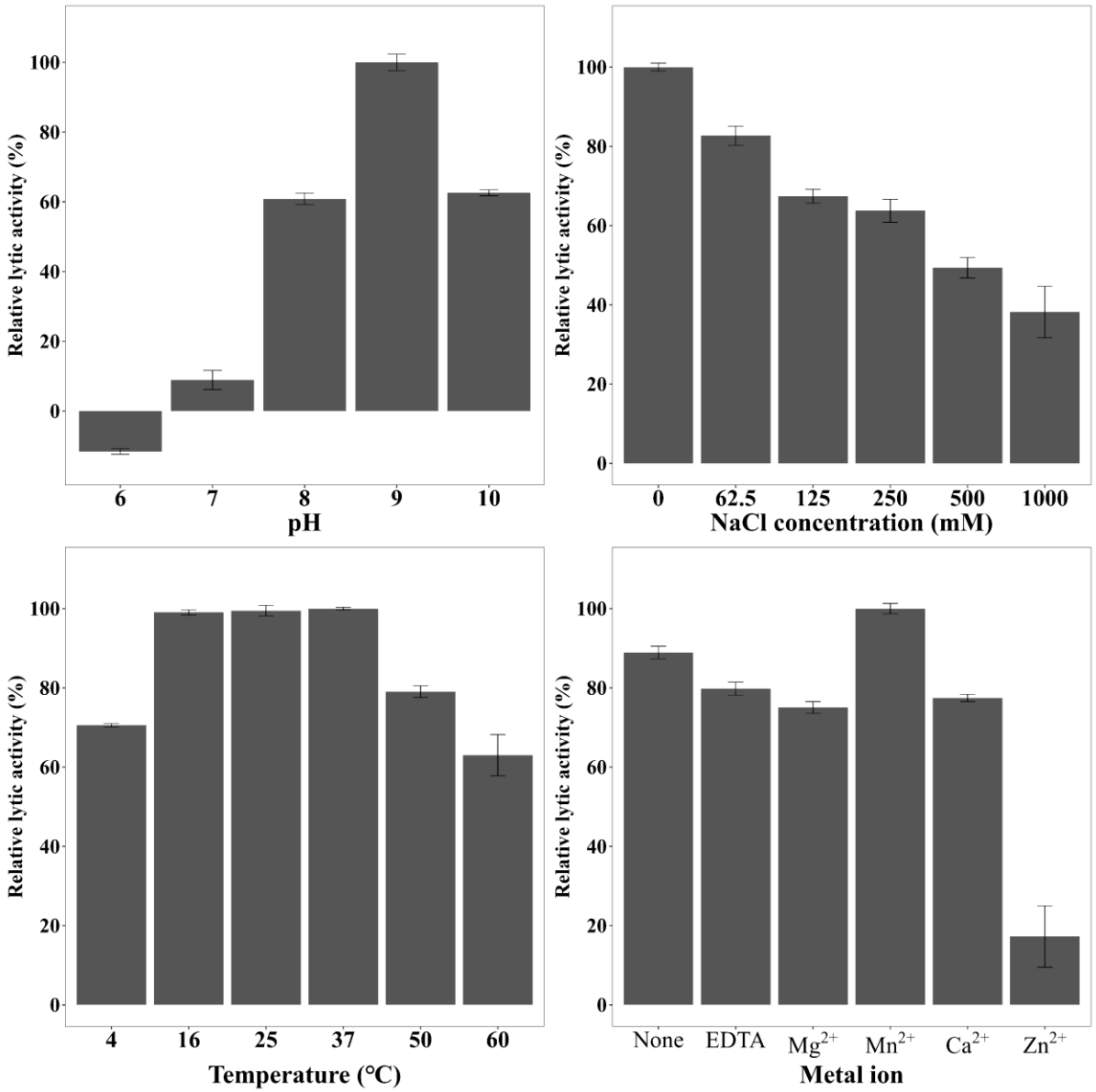


Fig. 3. Determination of the optimal conditions for SALys78925 lytic activity. *Streptococcus agalactiae* KCCM 40417 was treated with SALys78925 (final concentration: 100 μg/mL) at various (A) pH range, (B) NaCl concentrations, (C) temperatures, and (D) metal ions. In figure (D), "None" refers to purified SALys78925; "EDTA" represents SALys78925 incubated with 5 mM ethylenediaminetetraacetic acid (EDTA); and "Metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, or Zn^{2+})" indicate SALys78925 treated with 10 mM metal ions to EDTA-treated SALys78925. Each experiment was conducted in triplicate, with results expressed as mean ± standard error. Bars labeled with different superscript letters (a-e) denote a significant difference ($p < 0.05$).

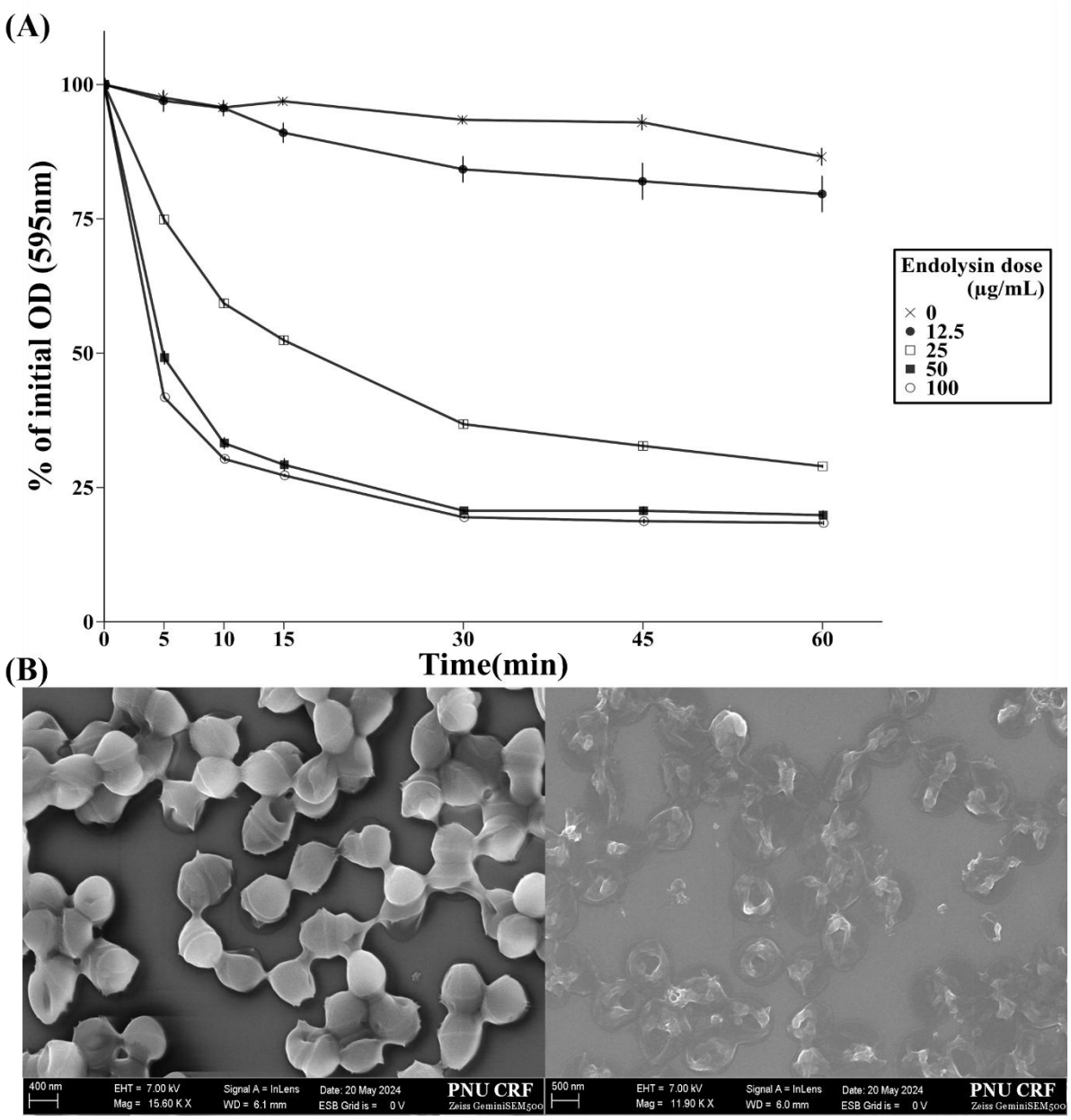
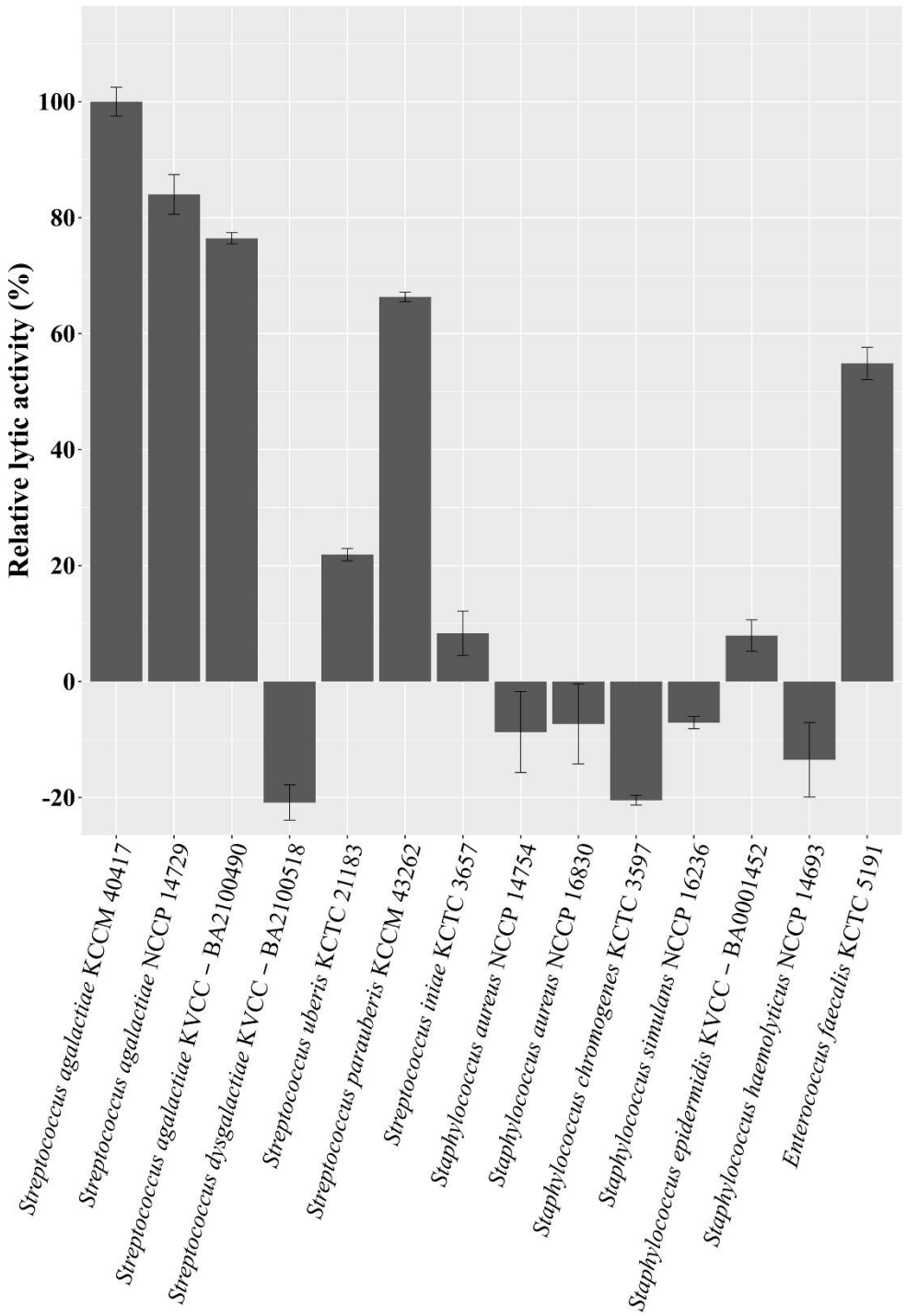


Fig.4. Dose-response test and scanning electron microscopy image of the lytic activity of SALys78925 against *Streptococcus agalactiae* KCCM 40417. (A) Dose-response test against *S. agalactiae* KCCM 40417. *S. agalactiae* was incubated with various doses of SALys78925 or elution buffer. (B) Scanning electron microscopy analysis of lytic activity of SALys78925 against *S. agalactiae* KCCM 40417. Log phase cells of strain *S. agalactiae* were treated with 100 μg/mL of SALys78925 with 1 h (right panel) or with elution buffer (left panel) and then visualized using scanning electron microscopy. Scale bar corresponds to 450-500 nm. All data represent the mean ± standard error from triplicate experiments. OD, optical density.



535

536 **Fig. 5. Antimicrobial spectrum of SALys78925.** Relative lytic activity (%) was calculated as the ratio of lytic

537 activity against each tested bacterial strain to the lytic activity against *S. agalactiae* KCCM 40417. All data

538 represent the mean ± standard error from triplicate experiments.

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