African Journal of Biotechnology Vol. 11(20), pp. 4649-4659, 8 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2430
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Lysis of mastitis pathogens isolated from dairy cow milk samples by purified recombinant lysostaphin

Bowei Zhang, Tao Shangguan, Huiming Ma, Xin Huang and Yong Zhang*

College of Veterinary Medicine, Northwest A & F University, Key Laboratory of Animal Reproductive Physiology and Embryo Technology, Ministry of Agriculture, Yangling, Shaanxi, 712100, PR China.

Accepted 23 February, 2012

To avoid the use of broad spectrum antibiotics which can induce antibiotic resistance, we developed a recombinant lysostaphin with high purity against mastitis pathogens isolated from dairy cow milk. The gene encoding for mature lysostaphin peptide was cloned into an *Escherichia coli* expression systems, pET28a, to produce recombinant lysostaphin. This recombinant lysostaphin was purified by a His60 Ni Gravity Column to obtain milligram amounts of recombinant lysostaphin protein with high purity. Staphylococcal mastitis pathogens, which were isolated from 30 milk samples from cows diagnosed with mastitis, were identified on the basis of colony characteristics, Gram staining, pigment production, hemolysis on 5% bovine blood agar and tube coagulase test. The antibiotic resistance of the isolates was determined by disc diffusion assay. Most of the tested isolates were resistant to some antibiotics. All the tested strains were lysed by the purified recombinant lysostaphin in turbidity reduction assay. The recombinant lysostaphin was found capable of killing *Staphylococci* pathogens *in vitro* within an hour (minimum inhibitory concentration (MIC) 2.5 to 16 µg/ml) and was active in a milk-like environment. Thus, the recombinant lysostaphin from the pET system is effective and is expected to complement clinical treatment of cow mastitis.

Key words: Lysostaphin, *Staphylococcus aureus*, bovine mastitis, expression, purification.

INTRODUCTION

Bovine mastitis is caused by environmental pathogens that enter the mammary gland through the teat canal when the animal is exposed to contaminated soil or bedding or during the milking process. Economic losses due to mastitis are estimated to be about two billion dollars annually in the U.S. dairy industry alone (Sordillo and Streicher, 2002). Treatment of mastitis has historically been limited to the use of broad-range antibiotics such as tetracycline and penicillin, which are often less than 50% successful (Deluyker et al., 2005). In addition, it is universally accepted that the use of broad-range antibiotics in dairy herds for mastitis treatment can lead to both antimicrobial resistance among mastitis pathogens and premature culling (Erskine et al., 2002; Rajala-Schultz et al., 2004). In two studies, 44% of 202 bacterial isolates and 57% of 811 isolates from bovine mastitis exhibited resistance to at least one antibiotic (De Oliveira et al., 2000; Rajala-Schultz et al., 2004). The anti-microbial resistant strains of mastitis pathogens might find their way from the farm to the clinic (Ferber., 2003). Over the past 30 years, the once-common mastitis pathogens, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*, have been eliminated from many herds by persistent implementation of the five-point control plan (Bramley and Dodd, 1984). However, *Staphylococcus aureus,* which is responsible for 15 to 30% of the infections, has proved more difficult to be inactivated (Sutra and Poutrel., 1994). As a result, the search for new antimicrobial agents is essential.

Lysostaphin (Lss), a zinc metalloproteinase extracted from *Staphylococcus simulans biovar staphylolyticus,* can cleave the cross-linking pentaglycine bridge in the cell wall of almost all known staphylococcal species (Schindler and Schuhardt, 1964, 1965). *S. aureus* cell walls contain high proportions of pentaglycine, making

*Corresponding author. E-mail: zhy1956@live.cn. Tel: +86 029 87080085. Fax: +86 029 87080085.

lysostaphin a highly effective agent against both actively growing and quiescent bacteria. The specificity of the agent to the pentaglycine bridge is very high: staphylococcal strains mutated in this region showed significantly decreased susceptibility to lysostaphin (Maidhof et al., 1991; De Jonge et al., 1993). The wild-type lysostaphin gene encodes a preproenzyme (493 aa), which consists of three distinct domains. It has a typical secretion signal peptide of 36 amino acid residues at the N-terminus, a propeptide of 211 amino acids from which 195 amino acids are organized in 15 tandem repeats of 13 amino acids, and finally a hydrophobic mature lysostaphin of 246 amino acids. The catalytic activity of the mature 27 kDa lysostaphin is approximately 4.5 times more intense than that of the precursor protein (Thumm and Gotz., 1997). The unique biological activity of lysostaphin presents numerous possibilities for application in the medical and veterinary fields, in the food industry, and in research. The gene coding lysostaphin has been cloned and expressed in *Lactobacillus curvatus* and *Lactobacillus sake*. The resulting strains were successfully used to kill staphylococci in fermented sausages and mayonnaise-based salads (Cavadini et al., 1996). Lysostaphin purified from *S. simulans* has been found to be effective in treating experimental staphylococcal infections in various animal models (Dixon et al., 1968; Zygmunt and Tavourrmina, 1972). It was once used systemically in a human neutropenic patient to treat staphylococcal abscesses (Stark et al., 1974).

Although the antimicrobial properties of lysostaphin appear promising, the lack of an efficient source of this protein, difficulties in the purification process, and the wide availability of alternative anti-staphylococcal antibiotics stopped further development of lysostaphin as a therapeutic agent more than 20 years ago. At present, several lysostaphin overexpression systems have been described (Williamson et al., 1994; Chan, 1996; Szweda et al., 2001). However, the yield remains unsatisfactory and purification methods are complicated.

In the present study, we described a new expression system for producing recombinant lysostaphin (rlysostaphin) in *Escherichia coli* and a high-capacity Ni-IDA resin purification procedure for obtaining milligram amounts of pure rlysostaphin. We then examined the *in vitro* activity of rlysostaphin against mastitis pathogens isolated from the milk samples of cows suffering from mastitis.

MATERIALS AND METHODS

Bacterial strains, vectors and other regents

All the molecular-biology-grade reagents and polyclonal antibodies were purchased from Sigma (Ronkonkoma, NY, USA). Primer synthesis and DNA sequencing were performed by Sangon (Shanghai, China). pET28a (+) vector and *E. coli* BL21 (λDE3) was purchased from Novagen (Germany). Restriction enzymes, DNA ligase, and *Pfu* DNA polymerase were obtained from Fermentas (Lithuania). His60 Ni Gravity Column Purification Kits (Cat. No. 635658) were bought from Clontech (TaKaRa, Mountain View, CA, USA). Plasmid Prep DNA Kit and DNA Fragment Purification Kit were provided by Qiagen (China). Blue Plus II Protein Marker was bought from TransGen Biotech of Beijing (China). Mastitis pathogens isolated from mastitis bovine milk samples were preserved in the Microbiology Laboratory of Animal Medicine of Northwest A & F University. Quality control strains (*S. aureus* (ATCC25923)) were provided by the China Institute of Veterinary Drug Control (Beijing, China).

Construction of the rlysostaphin-expressing plasmid

The full-length of lysostaphin gene (GeneBank accession no. M15686) was synthesized by TaKaRa Bio Incorporated and cloned into PUC19 vector. The coding sequence of the mature peptide (738 bp) was amplified by polymerase chain reaction (PCR) from PUC-lys using the following primers: sense 5´-CATGCCATGGCTGCAACACATGAACATTC-3´ (forward primer with an endonuclease site *Nco*I) and antisense 5´-CCGCTCGAGCTTTATAGTTCCCCAAAGAACA-3´ (reverse primer with an endonuclease site *Xho*I) (Heinrich et al., 1987). Restriction endonuclease sites for *Nco*I and *Xho*I were incorporated at the 5´-end and 3´-end of the mature gene, respectively, for sub-cloning purposes. An initiator methionine codon (ATG) in restriction endonuclease sites for *Nco*I is the starting of mature lysostaphin.

50 µl of reaction solution consisted of template DNA 2 µl (10 ng), forward and reverse primer 2 µl each (20 µmol), dNTPs Mix 5 µl (12.5 mM), *pfu* DNA polymerase 0.4 µl (1U),10× *pfu* buffer with Mg^{2+} 5 µl. PCR was performed as follows: 95°C for 5 min, 30 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 30 s, and then 72°C for 10 min. The PCR product was purified by gel elution, digested with *Nco*I and *Xho*I restriction endonucleases, and cloned into the digested expression vector pET28a (+) to generate the recombinant plasmid pET28a-lys (pET-lys) using T4 DNA ligase. The lysostaphin gene of the recombinant plasmid was sequenced by BGI (Beijing, China).

Expression of recombinant mature lysostaphin with 6× His tag

A single colony of *E. coli* BL21 (λDE3) transformed with pET-lys was grown overnight in 5 ml Luria-Broth (LB) medium (1% NaCl, 1% tryptone, 0.5% yeast extract, pH 7.0) containing 50 µg/ml kanamycin at 37°C with constant agitation (200 rpm). 200 µl of overnight-grown culture was inoculated into 20 ml of LB containing 50 µg/ml kanamycin. The culture was grown in an OD_{600nm} of 0.6 to 0.8 with vigorous shaking (200 rpm) at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for expression of mature lysostaphin in the cytoplasm of *E. coli*. The incubation continued for another 10 h at 25°C with shaking at 180 rpm. Every 2 h, a 1.5 ml bacterial suspension was taken and centrifuged at 5000 rpm for 15 min at 4°C to remove the supernatant. The cells were washed twice with phosphate-buffered saline (PBS) and then resuspended in 100 µl PBS. Then 100 µl 2× sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample/loading buffer was added to the cell suspension. The mixture was boiled at 100°C for 5 min and centrifuged to remove the cell debris. The soluble fraction were analyzed on a 12% SDS-PAGE gel and stained with Coomassie brilliant blue to determine the expression level of the recombinant protein.

Purification of the rlysostaphin

For purification, 1 L of cell culture was incubation at 25°C for 6 h in

the presence of 1.0 mM IPTG and the cells were harvested by centrifugation. The pellet (about 8 g) was re-suspended in 50 mM phosphate buffer (pH 7.4) (containing 500 mM NaCl, 40 mM imidazole and 1 mM phenylmethylsulfonyl fluoride (PMSF)), broken by sonication, and then centrifuged at 4°C and 10,000 g for 20 min. Subsequently, the supernatant was filtered with a 0.45 µm filter membrane to remove the remaining cell debris. The filtered lysate was passed through an His60 Ni Gravity Column (Clontech) equilibrated in advance with His60 Ni equilibration buffer (50 mM PBS, 300 mM NaCl, and 20 mM imidazole, pH 7.4). The column was washed with 10 column volumes of His60 Ni wash buffer (50 mM sodium phosphate, 300 mM NaCl, 40 mM imidazole, pH 7.4) to remove contaminating proteins and the recombinant fusion proteins were eluted with approximately 10 column volumes of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole, pH 7.4). The eluted fractions were collected and analyzed by analytical SDS-PAGE electrophoresis.

Western blot analysis

The purified recombinant lysostaphin protein sample was electrophoresed on two 12% SDS-PAGE gels and transferred to a polyvinylidene difluoride (PVDF) membrane for 30 min (15 V) using a Bio-Rad transfer apparatus. Then the membrane was blocked overnight at 4°C with 5% (wt/vol) milk in Tris-buffered saline with 0.1% Tween-20 (TBST) buffer. Membranes were incubated with anti-rabbit His tag antibody (1:2000 dilution in TBST with 1% milk) for 2 h at room temperature according to the standard protocol. After being washed five times with TBST, the membrane was incubated with a secondary anti-rabbit IgG–horseradish peroxidase conjugate (1:5000 dilution in TBST) for 1 h at room temperature. Finally, the membrane was washed three times and the specific protein bands were visualized with the enhanced chemiluminescence (ECL) system.

Isolation and identification of microorganisms

30 mastitic milk samples were collected from several dairy farms in the Yangling District, Shaanxi Province, China. In a sterile environment, two or three rings of milk samples were taken with a sterile inoculation loop and plated on an ordinary agar plate. The plate was incubated at 37°C for 24 h and then colony morphology was evaluated. Samples with morphologies consistent with *Staphylococcus* were transferred to tryptic soy agar, streptokokken agar, 5% sheep blood agar and mannitol salt agar, respectively. After growth, *Staphylococci* sp. were identified on the basis of colony characteristics, Gram staining, pigment production, hemolysis on 5% bovine blood agar, and tube coagulase testing. *Staphylococcus* sp. were classified as described by (Espinola and Lilenbaum ., 1996). One colony from each isolate was transferred to stock agar.

Susceptibility tests

All strains isolated were tested for susceptibility to antimicrobial agents by the agar disc diffusion method on Mueller Hinton agar at 37°C. Discs of penicillin G (10 units), ampicillin (10 µg), oxacillin (5 µg), gentamicin (10 µg), amoxycillin (10 µg), vancomycin (50 µg), tetracycline (10 µg), cephalexin (30 µg) and rifampin (10 µg) were used. After measuring the diameters of antimicrobial zones and following the instructions of the manufacturer of the antibiotic discs, the strains were categorized as susceptible or resistant to each drug. Methicillin resistance was determined by the agar screen method in Mueller-Hinton agar supplemented with 4% NaCl and oxacillin (6 mg l^{-1}) as previously described (Standards, 2011). Data were compiled and analyzed by a x^2 test.

Turbidity assay

The turbidity assay measures a decline in optical density due to lysis of the target bacterium with the phage protein. Mid log phase (OD_{600nm} of 0.4 to 0.6) *S. aureus* (ATCC25923) was grown at 37°C in nutrient broth supplemented with 0.5% NaCl for 18 to 24 h and then concentrated in the activity buffer (50 mmol/l Tris-HCl, pH 8.0, 100 mol/l NaCL) to an OD_{600nm} of 1.2 (Sharma et al., 2006). Turbidity assays were performed with 25 µg of lysostaphin (Sigma), His-tag protein, and the purified rlysostaphin, respectively. The reaction mixture, containing 6 ml of cell suspension and 25 µg protein, was incubated at 37°C. After 10 min, changes in turbidity of the reaction mixture were assessed. One unit (U) of rlysostaphin activity was defined as the amount of preparation capable of causing a 50% reduction in turbidity of a 6-ml cell suspension within 10 min at 37°C in a 10-mm cuvette. The effect of pH on rlysostaphin activity was determined using 0.1 M phosphate buffers in the range of 5.0 to 9.0. The influence on rlysostaphin activity of Ca ions at concentrations of 2 mM and 10 mM (in 10 mM Tris–HCl, pH 7.5, 0.2 M NaCl buffer) was also determined by adding $CaCl_2$ at the appropriate concentrations. Turbidity reduction assays were performed in triplicates.

To further test rlysostaphin activity, we ordered three mastitis-causing strains of *Streptococci* (CVCC1886, CVCC587 and CVCC593) and one strain of *E. coli* (ATCC25922) from the China Institute of Veterinary Drug Control. In addition, five strains of *Staphylococcus* were randomly selected from different isolated *Staphylococcus* sp. Turbidity assays were performed with 25 µg of purified rlysostaphin protein in a cuvette. The assays were initiated by adjusting the target cell suspension to a final $OD_{600\ nm}$ of 2.0 at room temperature. Changes in OD were recorded for 1 h ($OD_{600\ nm}$).

The minimum inhibitory concentration (MIC) for the purified recombinant lysostaphin (rLYS) was determined using the micro broth-dilution method. Purified recombinant lysostaphin was diluted from 0.5 to 10 µg/µl in phosphoric acid buffer (pH 8.0) and added to sterile 96-well microtiter plates (COSTAR). Approximately 10^5 colony-forming units (CFU)/ml of bacteria to be tested were added to the 96-well microtiter plates and incubated at 37°C for 18 to 24 h. For wells that served as the non-treated contrasts, no drug was added. The MIC_{90} was defined as the lowest concentration capable of inhibiting 90% of the microorganism tested according to the criteria described by Clinical and Laboratory Standards Institute. After the MIC was determined, one to three wells of the rLYS reaction mixture near MIC were plated on broth-agar plates and cultured at 37°C for 16 h. The minimum bactericidal concentration (MBC) of rLYS was then determined.

RESULTS

Construction of expression plasmid

Figure 1 shows nucleotide and amino acid sequences of pET-lys plasmid near the site of lysostaphin gene insertion. The genetic construct retained an open reading frame for rlysostaphin.

Expression of rlysostaphin

The positive recombinant plasmid was transformed into the host, *E. coli* BL21 (λDE3). The addition of IPTG

Figure 1. Nucleotide and amino acid sequences of pET-lys plasmids near the site of mature lysostaphin gene insertion. The amino acid sequence of the lysostaphin is in bolded

induced the overexpression of a band of approximately 27 kDa molecular weight of the rlysostaphin protein (Figure 2). The results obtained reveal that 6 h of induction at 25°C led to a substantial overproduction of the rlysostaphin protein (Figure 2, lane 5).

Purification of rlysostaphin and Western blot analysis

The rlysosotaphin was eluted from the column using a buffer containing 300 mM imidazole. The purity of rlysostaphin was estimated to be 96% by SDS-PAGE with Coomassie brilliant blue staining (Figure 3); and was determined by immunoblot analysis (Figure 4). The purification process resulted in a yield of about 22 mg of purified protein from 1 L of *E. coli* BL21(λDE3) + pET-lys culture by optical density (OD) determination (Table 1).

Isolation and identification of *Staphylococci*

50 isolates with the typical characteristic of the genus *Staphylococcus* were obtained from 30 milk samples. Coagulase-positive species of *Staphylococci* were the most common, representing 52% of the isolates. The most frequently isolated *Staphylococcus* sp. (26 samples) was *S. aureus*. Other coagulase-negative species, such as *Staphylococcus epidermidis*, *Staphylococcus xylose*, *Staphylococcus saprophyticus,* and *Staphylococcus human*, were isolated in 14, five, three, and two species, respectively. The frequency of isolation of the different staphylococcus species is shown in Table 2.

Susceptibility tests

The results of the antimicrobial susceptibility tests are given in Tables 3 and 4. As seen in Table 3, the more sensitive antibiotics against the 50 isolates were gentamicin, vancomicin, and tetracycline, which have susceptibility rates of 96, 98, and 84%, respectively. Resistance to antibiotics was frequently observed in Table 4, with 46% of the isolates (23 samples) showing resistance to at least one drug. The resistance of *S. aureus* to oxacillin indicates that they are methicillin resistant *S. aureus* (MRSA). These MRSA strains had been tested for the *mec* cassette (data not shown). Resistance to penicillin G was common (observed in 18 (36%) of the 50 isolates). Coagulase-negative strains (41.6%) were more frequently resistant to penicillin G than coagulase-positive strains (34.6%). 16 samples were resistant to ampicillin and oxacillin (32% of isolates), 14 to amoxicillin, seven to cephalexin, and five to tetracycline. The active antimicrobial agent against *Staphylococci* isolated from mastitic milk samples were vancomycin and gentamicin, with one and three samples, respectively, showing resistance (2 and 6%) to these drugs.

Activity of rlysostaphin and influence of pH and calsium ion concentration on activity of rlysostaphin *in vitro*

As shown in Figure 5, the specific lytic activity of rlysostaphin to *S. aureus* (ATCC25923) is similar to that of the commercial lysostaphin (Sigma). However, His-tag proteins have no specific lytic activity to *S. aureus* (ATCC25923) as indicated by no change of OD_{600nm} (Figure 5). The physiological pH and free calcium concentration of bovine milk is 6.7 and 3 mM, respectively (Pritchard et al., 2004).

According to Figure 6, the staphylolytic activity of rlysostaphin had slight affect on the turbidity assay when calcium ion concentration was adjusted from 2 to 3 mM. The rlysostaphin is active at a broad range of physiologically relevant pH (Figure 7). At 37°C and the physiological pH of bovine milk, the rlysostaphin displayed 50%

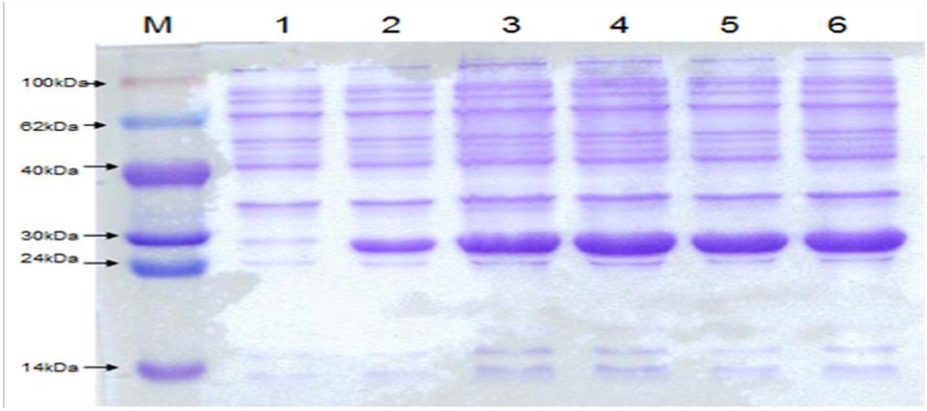

Figure 2. SDS-PAGE gels with Coomasssie brilliant blue staining showing the expression of recombinant lysostaphin in pET system. The SDS-PAGE gels showed uninduced cell extract from *E. coli* BL21 (λDE3)+ pET-lys (lane 1) and induced cell extract for 2 h (lane 2), 4 h (lane 3), 6 h (lane 4), 8 h (lane 5) and 10 h (lane 6). A high-range molecular weight marker is shown on the left (lane M).

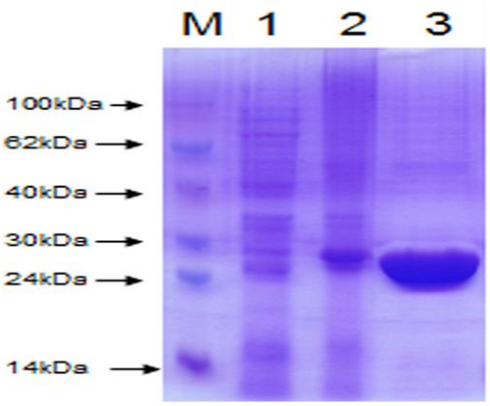

Figure 3. Purified (His)6–lysostaphin protein on SDS-PAGE with Coomassie blue staining. Lane M, Molecular weight marker; lane 1, uninduced cell extract from *E. coli* BL21 (λDE3)+ pET-lys; lane 2, induced cell extract from *E. coli* BL21 (λDE3)+ pET-lys; lane 3, purified His-lysostaphin fusion protein.

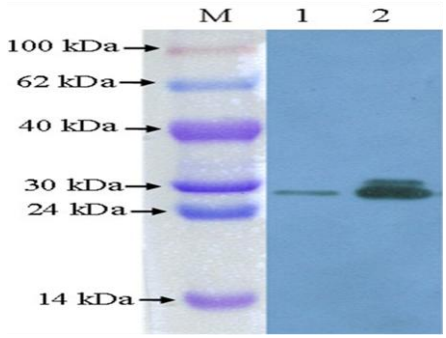

Figure 4. Western blot of purified rlysostaphin. Lane M, Molecular weight marker; lane 1, induced cell extract from *E. coli*; lane 2, purified rlysostaphin protein.

Table 1. Purification of lysostaphin expressed in *E. coli* (1 L of induced culture).

Purification	Total protein (mg)[a]	Specific activity (u/mg)	Protein purity (%)[b]	Purification fold
Total bacterial lysate	ND	ND	1	ND
Cleared lysate	250	ND	7	1
His60Ni gravity column	∽11	630	98	18

ND, not determined; [a]concentration of proteins in the cleared lysate as determined by Pierce BCA assay using BSA as a standard, the concentration of protein after purification was determined using the Bradford dye binding assay; [b]enzyme purity was estimated by analyzing images of the SDS-PAGE gel stained with Coomassie blue using Biometra ScanPack software. SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; BCA, bicinchoninic acid.

Table 2. Staphylococcal species isolated from 30 milk samples

Species	Coagulase	Number of isolate	Percentage (%)
Staphylococcus aureus	Positive	26	52
Staphylococcus epidermidis	Negative	14	28
Staphylococcus xylose	Negative	5	10
Staphylococcus saprophyticus	Negative	3	6
Staphylococcus human	Negative	2	4
Total		50	100

Table 3. Susceptibility of staphylococci isolates to nine antibiotics.

Drug	Chemical group	Susceptibility rate (%)	Number of susceptible isolate
Penicillin G	β-lactam	46%	23
Ampicillin	β-lactam	46%	23
Oxacillin	β-lactamase-resistant	78%	39
Gentamicin	aminoglycoside	96%	48
Amoxycillin	β-lactam	44%	22
Vancomycin	macrolide	98%	49
Tetracycline	tetracyclines	84%	42
Cephalexin	cepholosporin	40%	20
Rifampin	Macrocyclic	88%	44

of its maximum activity.

Bacteriolytic activity of rlysostaphin towards different mastitis pathogens *in vitro*

Rlysostaphin was found to be lytic for five of the strains isolated from mastitis infections in turbidity assay. It was found not to be lytic for mammitis-causing pathogens of *Streptococci* (CVCC1886, CVCC587 and CVCC593) and one of *E. coli* strain (ATCC25922) (Figure 8). To further test the lytic activity of rlysostaphin, we determined the rlysostaphin concentrations that gave the strongest bacteriolytic activity and found that they varied according to species (MIC 2.5 to 16 µg/ml) (Table 5).

DISCUSSION

Antimicrobial peptides for the prevention of mastitis in cows have the potential to reduce the dependence on antibiotics in the future (Ryan et al., 1999). Lysostaphin, a peptidoglycan hydrolase, received a great deal of attention as an antimicrobial agent. Unlike an antibiotic, which interferes with bacterial growth, lysostaphin is highly effective in lysing *S. aureus* cells in all the metabolic stage. However, purification of wild-type lysostaphin is very difficult. Although several methods of lysostaphin production have reported (in previous studies) that the yield and purity are very limited (Marova and Dadak., 1993; Motoyuki et al., 1990; Szweda et al., 2001). Moreover Cibacron blue, involved in the production

Table 4. Antimicrobial resistance of *Staphylococci* species isolated from 30 milk samples.

Organism	Resistant sample	Antimicrobial resistance pattern (no. of isolates)
Staphylococcus aureus	9/26 (34.6%)	PN OX RF (1); PN AO AP (5); PN OX AO AP (1); PN AO (1); PN OX RF AO AP CP (1)
Staphylococcus epidermidis	5/14 (35.7%)	TT PN AP OX (1); TT AP OX (3); PN OX (1)
Staphylococcus xylose	4/5 (80%)	RF CP TT VN (1); PN OX AP (3)
Staphylococcus saprophyticus	3/3 (100%)	PN AP PX GN AO CP (3)
Staphylococcus human	2/2 (100%)	PN TT RF AP OX AO CP (2)
Total	23/50 (46%)	PN OX RF (1); PN AO AP (5); PN OX AO AP (1); PN AO (1); PN OX RF AO AP CP (1); TT PN AP OX (1); TT AP OX (3); PN OX (1); RF CP TT VN (1); PN OX AP (3); PN AP PX GN AO CP (3); PN TT RF AP OX AO CP (2)

PN, Penicillin G; AP, ampicillin; OX, oxacillin; GN, gentamicin; AO, amoxycillin; VN, vancomycin; TT, tetracycline; CP, cephalexin; RF, rifampin.

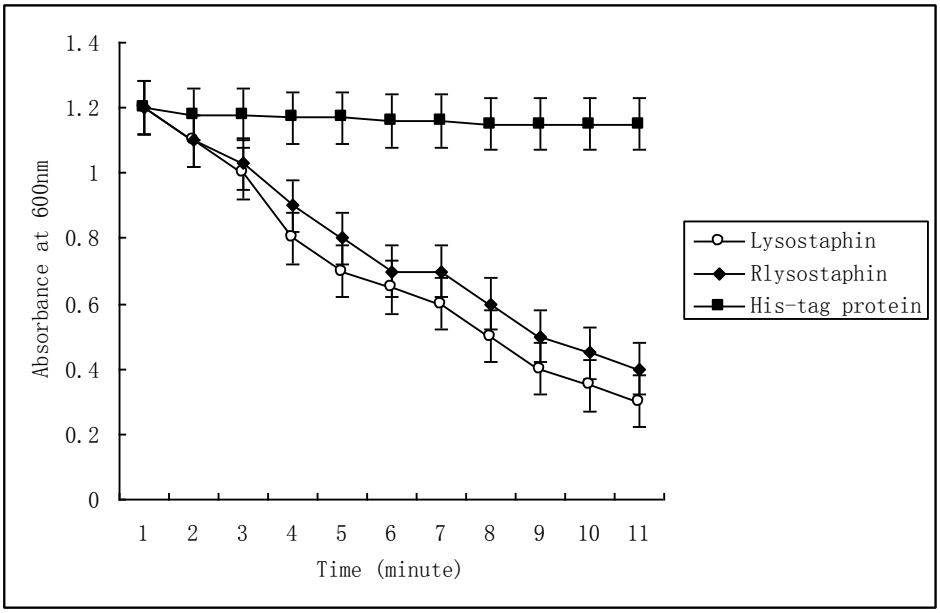

Figure 5. 16 µg of lysostaphin and rlysostaphin are active against ATCC25923. His-tag protein has no lytic activity in the turbidity assays against ATCC25923. Absorbance at 600 nm (OD 600nm).

process was found to be detrimental to lysostaphin activity (Marova et al., 1993).

E. coli is considered a safe expression host (as far as pharmaceutics/therapeutics) to produce numerous active recombinant proteins. Lysostaphin endopeptidase under the control of lysotaphin endopeptidase promoter has been expressed in *E. coli* (Recsei et al., 1987).

In the present study, we used the pET system to express the recombinant lysostaphin in *E. coli*. The results obtained show that it was very efficient. Using our purification methods, we obtained about 11 mg of recombinant lysostaphin per liter of the growth medium in the pET system. The yield is similar to that of the system described previously (Chan, 1996). However, the method of purification in Chan's study is cumbersome and time-consuming. In our assay, the recombinant lysostaphin was purified by a single His60 Ni Gravity Column. This purification method is very simple and performed in laboratories that have neither the expertise nor the equipment necessary for traditional protein purification schemes. Furthermore, the His60 Ni Gravity Column can be regenerated and used repeatedly. The procedure for producing rlysostaphin is quite convenient and efficient and would allow a laboratory to produce large amounts of

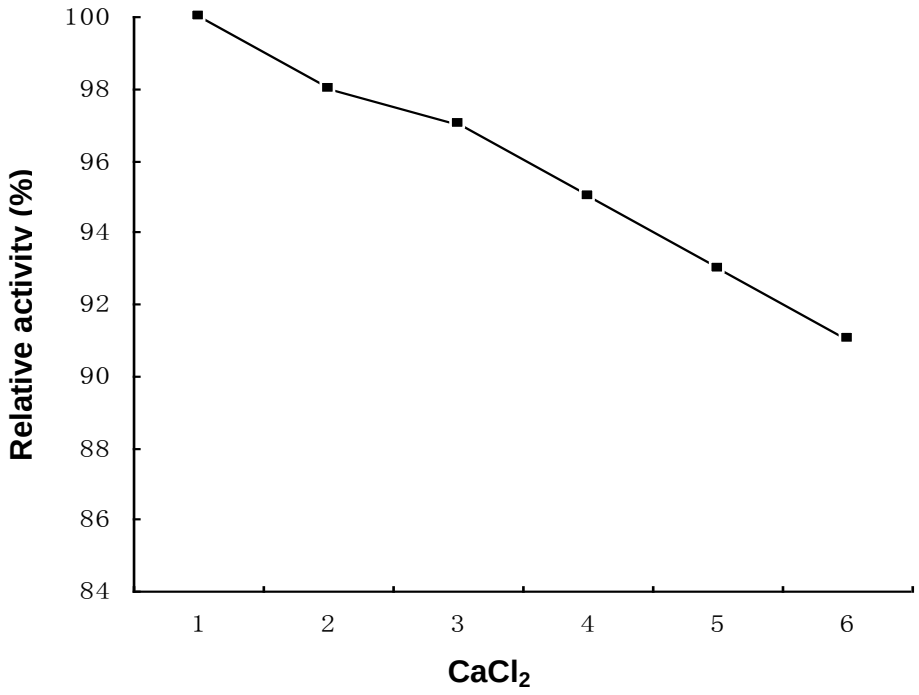

Figure 6. Effects of Ca ion concentration on the lytic activity of rlysostaphin (Graph shows a representative plot). The results are mean values of three replicates. Activity assay was performed by using ATCC25923 for 10 min at 37°C. Twenty-five micrograms rlysostaphin per sample were evaluated.

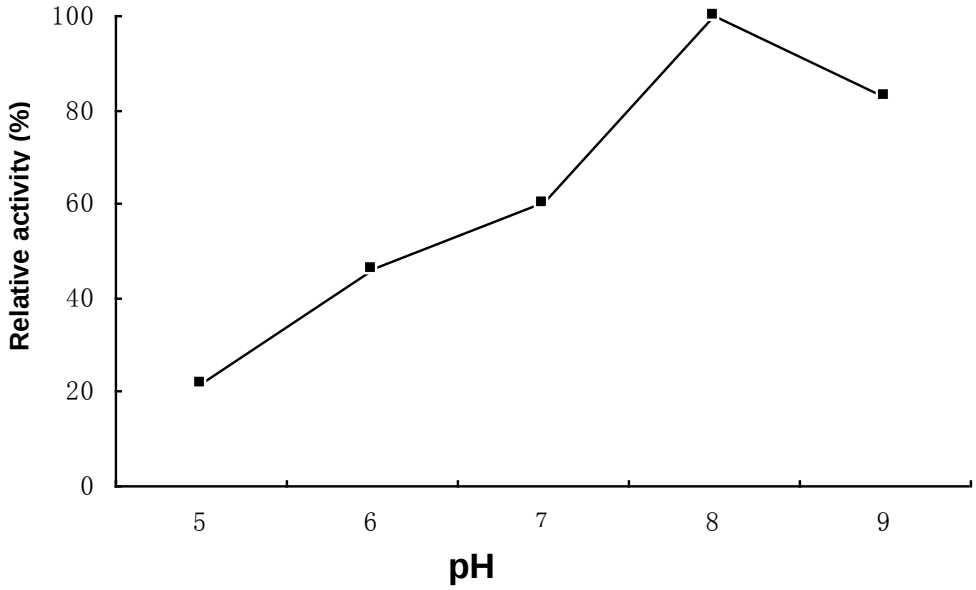

Figure 7. Effects of pH on rlysostaphin activity (graph shows a representative plot). Rresults are mean values of three replicates. Activity was performed by ATCC25923 for 10 min at 37°C. Twenty-five micrograms of rlysostaphin per sample were evaluated.

rlysostaphin. In the past, the systemic use of lysostaphin has not been encouraged because of immunogenicity from the previously impure proteins (Dajcs et al., 2000). However, the highly pure rlysostaphin proteins (>96%) were obtained by this purification and can be used in antibiosis studies.

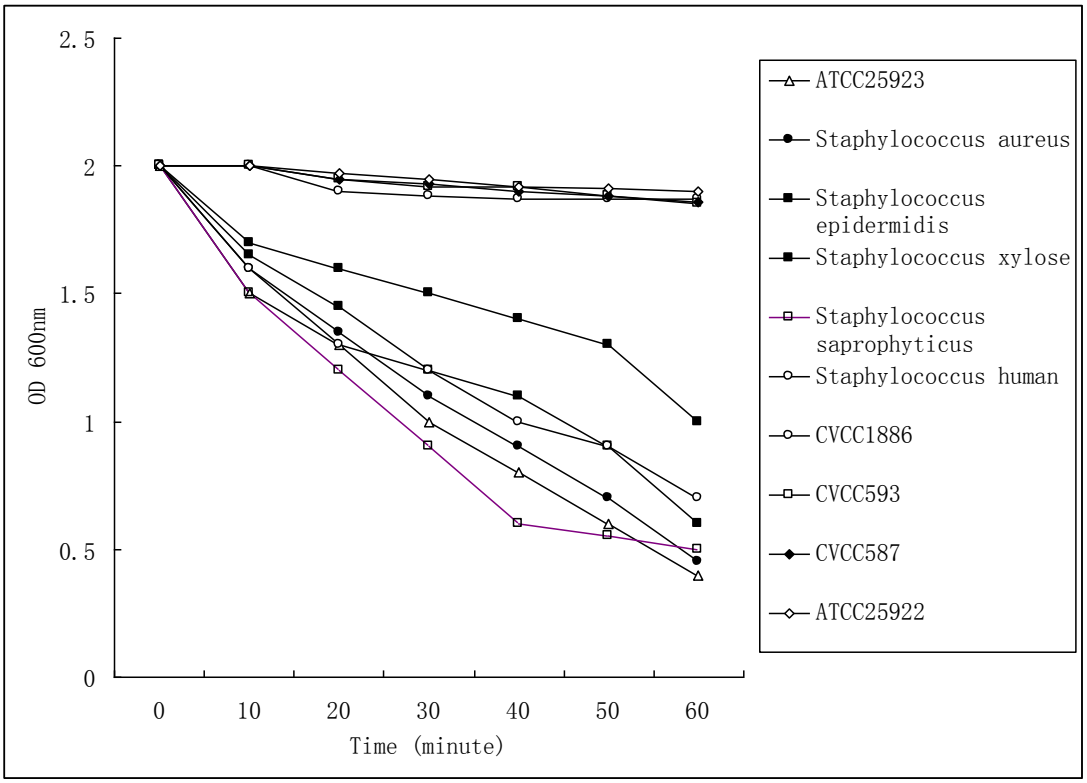

Figure 8. Turbidity assay results of rlysostaphin lysis of the different mastitis pathogens. 25 µg of rlysostaphin per sample were evaluated. Results are mean values of three replicates.

Table 5. Evaluation of the MICs of rlysostaphin for strains of different staphylococcus species isolated from bovine clinical mastitis.

Strain	Rlysostaphin (µg/ml)		
	MIC_{90}[a]	MBC	Range
ATCC 29213[b]	0.5		
Staphylococcus aureus (26)	2.5	32	0.04 - 32
Staphylococcus epidermidis (14)	16	68	2 - 68
Staphylococcus xylose (5)	14	16	8 - 16
Staphylococcus saprophyticus (3)	16	18	13 - 18
Staphylococcus human (2)	10	12	10 - 12

[a]Values indicate concentrations inhibiting all strains tested (90% MIC); [b]ATCC29213 is a quality control strain. MIC, Minimum inhibitory concentration; MBC, minimum bactericidal concentration.

In order to investigate the antimicrobial effect of the purified rlysostaphin we isolated *Staphylococcus* sp. from the milk samples of mastitis. Among these *Staphylococcus* sp. that caused bovine mastitis, *S. aureus* and coagulase-negative strains (accounting for 52 and 48% of the samples, respectively) were the major mastitis pathogens in our local dairy farm. Susceptibility tests showed that most staphylococcal species were resistant to penicillin G. However, coagulase-negative strains (41.6%) were more frequently resistant to penicillin G than coagulase-positive strains (34.6%). Their susceptibility to amoxycillin and ampicillin was not significantly different (44 and 46%, respectively). The results obtained indicate that the board-range antibiotics in the treatment of *Staphylococcus* mastitis would appear to be an ideal choice. However, the use of broad-range antimicrobials, although successful, is considered to cause antimicrobial resistance among mastitis pathogens (Werckenthin et al., 2001; Rajala-Schultz et al., 2004). Therefore, we use the purified rlysostaphin to lyse *S.*

aureus (ATCC25923). At the same time, the rlysoataphin activity was observed at pH 6.7 and free-calcium concentration (3 mM) was consistent with the milk. These results suggest that the recombinant lysostaphin was effective under *in vitro* conditions and its antimicrobial effect is almost the same as that of commercial lysostaphin (Sigma). However, the simple His-tag protein had no lytic activity against bacteria (Figure 5). Therefore, it would not affect the lytic activity of the mature lysostaphin fused by His-tag on C terminal. We further determined the antimicrobial activity of purified rlysostaphin *in vitro* by lysing *Staphylococcus* sp. from the milk samples of mastitis. Figure 8 shows that rlysostaphin had lytic activity against all tested *Staphylococcus* strains but no lytic activity against *Streptococci* or *E. coli*. It is further proved that rlysostaphin can only lyse staphylococci strains. Significantly, MIC for different mastitis pathogens show that rlysostaphin exhibited limited activity to the coagulase-negative staphylococci that cause bovine mastitis (Table 5). The results also highlight the potential for the use of rlysostaphin as an anti-mastitis agent. Although rlysostaphin is a large protein of approximately 27 kDa, its effectiveness in lysing *Staphylococci in vitro* indicates that large protein products can exert potent antimicrobial activity.

In summary, the recombinant lysostaphin purified from our methods demonstrates significant activity against the *Staphylococci* pathogens of mastitis under *in vitro* conditions. This could form the basis of an improved treatment for the prevention of mastitis in cows in near future. Further work on this research is to examine its antimicrobial effect in bovine mammary gland *in vivo.*

ACKNOWLEDGEMENT

This work was supported by a grant from the National Major Research Program involving Transgenic Breeding for Disease Resistance (Grant No. 2008ZX08007-004).

REFERENCES

Bramley AJ, Dodd FH (1984). Reviews of the progress of dairy science: mastitis control-progress and prospects. J. Dairy Res. 51(3): 481-512.

Cavadini C, Hertel C, Hammes WP (1996). Stable expression of the lysostaphin gene in meat lactobacilli by introducing deletions within the prosequence. Syst. Appl. Microbiol. 19(1): 21-27.

Chan EC (1996). Expression and purification of recombinant lysostaphin in *Escherichia coli*. Biotechnol. Lett. 18(7): 833-838.

Dajcs JJ, Hume EB, Moreau JM, Caballero AR, Cannon BM, O'Callaghan RJ (2000). Lysostaphin treatment of methicillin-resistant *Staphylococcus aureus* keratitis in the rabbit. Invest. Ophthalmol. Vis. Sci. 41(6): 1432-1437.

De Jonge BL, Sidow T, Chang YS, Labischinski H, Berger-Bachi B, Gage DA, Tomasz A (1993). Altered muropeptide composition in Staphylococcus aureus strains with an inactivated femA locus. J. Bacteriol. 175(9): 2779-2782.

De Oliveira AP, Watts JL, Salmon SA, Aerestrup FM (2000). Antimicrobial susceptibility of Staphylococcus aureus isolated from bovine mastitis in Europe and the United States. J. Dairy Sci. 83(4): 855-862.

Deluyker HA, Van Oye SN, Boucher JF (2005). Factors affecting cure and somatic cell count after pirlimycin treatment of subclinical mastitis in lactating cows. J. Dairy Sci. 88(2): 604-614.

Dixon RE, Goodman JS, Koenig MG (1968). Lysostaphin: an enzymatic approach to staphylococcal disease. 3. Combined lysostaphin-methicillin therapy of established staphylococcal abscesses in mice. Yale J. Biol. Med. 41(1): 62-68.

Erskine RJ, Walker RD, Bolin CA, Bartlett PC, White DG (2002). Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. J. Dairy Sci. 85(5): 1111-1118.

Espinola MB, Lilenbaum W (1996). Prevalence of bacteria in the conjunctival sac and on the eyelid margin of clinically normal cats. J. Small Anim. Pract. 37(8): 364-366.

Ferber D (2003). Antibiotic resistance - WHO advises kicking the livestock antibiotic habit. Science, 301(5636): 1027.

Heinrich P, Rosenstein R, Bohmer M, Sonner P, Gotz F (1987). The molecular organization of the lysostaphin gene and its sequences repeated in tandem. Mol. Gen. Genet. 209(3): 563-569.

Maidhof H, Reinicke B, Blumel P, Berger-Bachi B, Labischinski H (1991). femA, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible Staphylococcus aureus strains. J. Bacteriol. 173(11): 3507-3513.

Marova I, Dadak V (1993). Modified Simplified Method for Isolation of Lysostaphin from the Culture Filtrate of Staphylococcus-Staphylolyticus. Folia Microbiol. 38(3): 245-252.

Motoyuki S, Tomoko A, Yoichiro M, Eisaku I, Hidekazu S (1990). Rapid purification method of lysostaphin for analysis of cell-wall proteins. J. Microbiol. Methods, 12: 133-138.

Pritchard DG, Dong S, Baker JR, Engler JA (2004). The bifunctional peptidoglycan lysin of Streptococcus agalactiae bacteriophage B30. Microbiology, 150: 2079-2087.

Rajala-Schultz PJ, Smith KL, Hogan JS, Love BC (2004). Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. Vet. Microbiol. 102: 33-42.

Recsei PA, Gruss AD, Novick RP (1987). Cloning, sequence, and expression of the lysostaphin gene from Staphylococcus simulans. Proc. Natl. Acad. Sci. USA. 84(5): 1127-1131.

Ryan MP, Jack RW, Josten M, Sahl HG, Jung G, Ross RP, Hill C (1999). Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin. J. Biol. Chem. 3147(274): 37544-37550.

Schindler CA, Schuhardt VT (1964). Lysostaphin: A New Bacteriolytic Agent for the Staphylococcus. Proc. Natl. Acad. Sci. USA. 51(3): 414-421.

Schindler CA, Schuhardt VT (1965). Purification and Properties of Lysostaphin-a Lytic Agent for *Staphylococcus Aureus*. BBA-general Subjects, 97(2): 242-250.

Sharma R, Sharma PR, Choudhary ML, Pande A, Khatri GS (2006). Cytoplasmic expression of mature glycylglycine endopeptidase lysostaphin with an amino terminal hexa-histidine in a soluble and catalytically active form in *Escherichia coli*. Protein Exp. Purif. 45(1): 206-215.

Sordillo LM, Streicher KL (2002). Mammary gland immunity and mastitis susceptibility. J. Mammary Gland Biol. Neoplasia, 7(2): 135-146.

Standards CL (2011). Performance standards for antimicrobial susceptibility testing; 21th informational supplement, document M100-S21. Clinical and Laboratory Standards Institute, Wayne, PA.

Stark FR, Thornsvard C, Flannery EP, Artenstein MS (1974). Systemic lysostaphin in man-apparent antimicrobial activity in a neutropenic patient. N. Engl. J. Med. 291(5): 239-240.

Sutra L, Poutrel B (1994). Virulence Factors Involved in the Pathogenesis of Bovine Intramammary Infections Due to *Staphylococcus-Aureus*. J. Med Microbiol. 40(2): 79-89.

Szweda P, Pladzyk R, Kotlowski R, Kur J (2001). Cloning, expression, and purification of the *Staphylococcus simulans* lysostaphin using the intein-chitin-binding domain (CBD) system. Protein Exp. Purif. 22(3): 467-471.

Thumm G, Gotz F (1997). Studies on prolysostaphin processing and characterization of the lysostaphin immunity factor (Lif) of *Staphylococcus simulans* biovar staphylolyticus. Mol. Microbiol.

23(6): 1251-1265.
Werckenthin C, Cardoso M, Martel JL, Schwarz S (2001). Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus*, and canine *Staphylococcus intermedius*. Vet. Res. 32: 341-362.
Williamson CM, Bramley AJ, Lax AJ (1994). Expression of the lysostaphin gene of *Staphylococcus simulans* in a eukaryotic system. Appl. Environ. Microbiol. 60(3): 771-776.
Zygmunt WA, Tavormina PA (1972). Lysostaphin: model for a specific enzymatic approach to infectious disease. Prog. Drug Res. 16: 309-333.