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Enhancing the antimicrobial activity of *Sus scrofa* lysozyme by N-terminal fusion of a sextuple unique homologous peptide

Dewei Zhu[a,b,c], Guolin Cai[a,b,c], Xiaomin Li[a,b,c], Jian Lu[a,b,c,d,*], Liang Zhang[a,b,c]

[a] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China
[b] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China
[c] School of Biotechnology, Jiangnan University, Wuxi 214122, China
[d] Industrial Technology Research Institute of Jiangnan University in Suqian, 888 Renmin Road, 223800, Jiangsu, China

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ABSTRACT

Sus scrofa lysozyme (SSL), an important component of the pig immune system, is a potential candidate to replace antibiotics in feed. However, there is little antimicrobial activity of natural SSL against gram-negative bacteria, which limits its application. In this study, a unique peptide (A-W-V-A-W-K) with antimicrobial activity against gram-negative bacteria was discovered and purified from trypsin hydrolysate of natural SSL. This unique peptide was fused to natural SSL and the recombinant fused SSL exhibited improved activity against gram-negative bacteria. The N-terminal fusion likely increased the membrane penetrability and induced programmed bacterial cell death. The recombinant fused SSL also showed higher activity against some gram-positive bacteria with O-acetylation. By N-terminal fusion of the sextuple peptide, the anti-microbial activity, either to gram-positive or negative bacteria, of the recombinant SSL was higher than the fusion of only one copy of the peptide. This study provides a general, feasible, and highly useful strategy to enhance the antimicrobial activity of lysozyme.

1. Introduction

Lysozyme (EC 3.2.1.17), also known as muramidase or N-acetylmuramide glycanhydrolase, is a glycoside hydrolase that can damage bacterial cell walls by attacking peptidoglycans in the cell walls, especially of gram-positive bacteria, hydrolyzing the glycosidic bond that connects *N*-acetylmuramic acid with the fourth carbon atom of *N*-acetylglucosamine to cause cell lysis. Gram-negative bacteria are less susceptible to lysozyme because they have a more complex envelope structure. Lysozyme was first discovered by Fleming in 1922 (Spitznagel, 1984), and has been found in many different cells, tissues, and secretions of organisms (Hussain et al., 2015; Lee et al., 2015; Rocha et al., 2015; Xu et al., 2014). The three major lysozyme types found in the animal kingdom are designated chicken (c-type), goose (g-type), and invertebrate (i-type) types (Callewaert and Michiels, 2010). Of these, the hen egg white lysozyme (c-type) has been widely studied.

Abbreviations: SSL, *Sus scrofa* lysozyme; LP, longer peptide; SP, shorter peptide; HLH, helix-loop-helix; CD, circular dichroism; AFM, atomic force microscope (microscopy).

* Corresponding author at: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, China.
E-mail addresses: jlu@jiangnan.edu.cn, wdhpangzi@126.com (J. Lu).

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Due to its strong antibacterial potential, mainly against gram-positive bacteria, lysozyme has attracted tremendous attention for use in the preservation of various food items, as well as in pharmacy, medicine, and veterinary medicine. A lot of work has focused on extending the antimicrobial spectrum of lysozyme through chemical modification, such as lipohilization and glycosylation with palmitic acid (Liu et al., 2000). Nevertheless, the generality of these methods is limited due to their complex process, low efficiency, and non-hereditary nature. Recent efforts to broaden lysozyme's activity against gram-negative bacteria by peptide or protein fusion (Lu et al., 2010) have achieved considerable success.

Antibiotics have been used in feed for about 50 years to prevent or reduce the incidence of infectious disease, increase efficiency and growth rate, and treat clinically sick animals. Due to the increasing demand of pork in consumer's diet and the rising problems due to the excessive use of antibiotics, there is an urgent need to find alternative agents to replace antibiotics in feed for livestock, especially for pig (Zhu et al., 2015). Although hen egg white lysozyme is well studied and partially used in the food industry, the enzyme isolation methods are unfeasible to perform on a sufficient commercial scale to meet the large demand of the feed industry. In addition, lysozyme added as a feed supplement may be degraded by enzymes in the alimentary tract or attacked by the immune system of the animals. *Sus scrofa* lysozyme (SSL) from the pig stomach is part of the innate immune system (Jolles et al., 1989; Yu and Irwin, 1996) and exhibits pepsin resistance, allowing it to avoid degradation during feeding. Therefore, use of SSL is of great interest to replace antibiotics in the feed industry.

Like other c-type lysozymes, SSL is not active to gram-negative bacteria in its natural form. The heat denaturation of lysozyme allows greatly improved antimicrobial action towards gram-negative bacteria (Derde et al., 2014). When heated at 80 °C for 10 min, SSL showed improved activity to gram-negative bacteria. Based on this, we proposed that SSL has an anti-gram-negative bacteria domain or structure that is hidden in its natural form and is only exposed after heat treatment. To test this hypothesis, we used several proteases to digest the recombinant SSL in an effort to reveal the anti-gram-negative bacteria domain or module. Then, the obtained protein was fused to SSL to improve its antibacterial activity, and the antibacterial mechanisms of the recombinant SSL were evaluated.

2. Materials and methods

2.1. Microbial strains and culture conditions

E. coli BL21 (DE3) along with vector pET-28a(+) were used for the expression of recombinant SSLs expression in LB (Luria-Bertani) medium supplemented with 50 mg/L kanamycin. *Pichia pastoris* X-33 (Invitrogen, CA, USA) containing vector pPICZαA-SSL was cultured in BMGY or BMMY medium for SSL expression.

To test antimicrobial activity, several gram-negative and -positive strains were collected, including *E. coli* ATCC 10798, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* CMCC(B) 46117, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enteritidis* CMCC(B) 50335, *Micrococcus luteus* ATCC 4698, and *Bacillus licheniformis* and *Bacillus subtilis* strains isolated and stored in our laboratory. The above strains were cultured in TSB (trypticase soy broth) medium at 37 °C.

2.2. Antimicrobial tests

Antimicrobial activity test was performed according to the method of Pellegrini et al. (1996) with several modifications using the collected bacteria strains and tested individually. 0.2 mL of the bacteria culture (OD_{600} = 0.6, about 5×10^7 CFU/mL) was diluted with 0.4 mL TSB, and then mixed with 0.2 mL of purified SSL, SSL hydrolysates, or fusion SSL solutions (TSB as control). TSB with no enzyme was added into the control tube. The mixture was then incubated at 37 °C for 2 h, and then serially diluted (1:10–1:10000) in 10 mmol/L sodium phosphate buffer (pH 7.4) for plating. The TSB plate was incubated at 37 °C until colonies grew. The colonies were counted for antibacterial activity calculation using the formula of $\log N_0/N_1$, where N_0 was the number of colonies on the control plate and N_1 referred to the number present on the plate containing SSL. Each experiment was repeated three times, and the average value was used for calculation.

2.3. Isolation, purification, and identification of peptides with anti-gram-negative bacterial properties

SSL was expressed in *Pichia pastoris* X-33 (Invitrogen, CA, USA) and purified by affinity chromatography on a HisTrap HP 1 mL column (GE, CT, USA) (Zhu et al., 2015). The lyophilized SSL was dissolved in NaCl (0.03 mol/L, pH 7.0) to 70 mg/mL for digestion with pepsin and trypsin (Sigma-Aldrich, CA, USA). 20 mL of SSL solution was mixed with 4 mL of pepsin solution (50 mg/mL in 0.03 mol/L NaCl, pH 1.0), or 4 mL of trypsin (30 mg/mL in 0.1 mol/L Tris-HCl containing 0.03 mol/L NaCl, pH 8.0) and incubated at 37 °C overnight. Separately, SSL was hydrolyzed by pepsin followed by trypsin digestion according to the method of Mine (Mine et al., 2004), using the same dose of SSL as used in pepsin digestion. After digestion, the mixtures were centrifuged at 10000 × *g* for 15 min, and the soluble supernatants were collected for SDS-PAGE separation (Bradford, 1976) and antibacterial activity analysis.

The hydrolyzed SSL solution was filtered through a 0.22 μm millipore filter and then separated by size exclusion chromatography using a Superdex peptide 10/300GL column (GE, CT, USA) with a flow rate of 0.5 mL/min and 20 mmol/L phosphate buffer (pH 7.0). The antimicrobial activity of each collected fraction was tested as described above. The fractions with antimicrobial activity were further separated by reverse phase-HPLC Agilent 1100 series (Agilent Technologies Inc., CA, USA) with a Phenomenex luna C18 column (7.8 mm × 150 mm, particle size 5 μm; Waters Corporation, MA, USA). Sterile Milli-Q water with 0.1% trifluoroacetic acid (A) and acetonitrile with 0.1% trifluoroacetic acid (B) were used as the mobile phase. The system was operated at a 10 mL/min flow rate with a linear gradient of solvent B (5–50%) for 30 min, and the detection was monitored at 225 nm with a diode array detector. Fractions were concentrated for antimicrobial activity determination and identification.

The purified peptides were sequenced by LC–MS in positive ionization mode on a Waters Alliance Platform ZMD (Waters Corporation, MA, USA) with Phonomenex luna C18 (4.6 × 250 mm) column. The mass spectrometer conditions were: capillary 3.88 kV, cone 20 V, ion source temperature 120 °C, desolvation temperature 300 °C, flow rate 1 mL/min, and split ratio 50:1. Mass Lynx software (version 4.1) was used to perform spectral processing and peak list generation for the MS spectra.

2.4. Gene synthesis and plasmid construction

One or six copies of peptide A-W-V-A-W-K coding sequences were added to the N- or C-terminal of SSL gene (the GenBank accession numbers of synthesized SSL gene and the fused genes were BankIt1948642 Seq1 KX806644, BankIt1948642 Seq2 KX806645, BankIt1948642 Seq3 KX806646, BankIt1948642 Seq4 KX806647 and BankIt1948642 Seq5 KX806648, respectively), with *Bam*H I and *Hin*d III cutting site added to two terminals of constructed genes, and were synthesized (Sangon Biotech (Shanghai) Co., Ltd.,