Letter to the Editor

Expression of Recombinant Human Lysozyme-tachyplesin I (hLYZ-TP I) in Pichia Pastoris and Analysis of Antibacterial Activity*

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Antimicrobial peptides (AMPs) are making headlines in science because they demonstrate superior microbicidal characteristics compared to synthetic and semi-synthetic antibiotics[1]. Importantly, AMPs kill pathogens via a different mechanism compared to antibiotics[2]. Therefore, AMP is one of the most promising anti-microbial drugs which could respond to the current problem of growing drug resistance.

Tachyplesin I, a cationic antimicrobial peptide, was found in the hemocytes of Japanese horseshoe crabs (Tachypleus tridentatus) as an inhibitor of the bacterial lip polysaccharide (LPS)-mediated activation of factor C, the initiation factor in the Limulus clotting cascade[3]. It inhibits the growth of Gram-positive and Gram-negative bacteria (Nakamura) as well as MRSA (*multidrug resistant staphylococcus aureus*) and fungi[4].

Lysozyme naturally exists in the milk, saliva, and tears of most mammals as well as in avian egg whites[5]. Human lysozyme catalytically hydrolyzes cell wall peptidoglycan and has also been shown to have catalysis-independent antimicrobial properties[6]. As the bactericidal mechanism is different from that of the chemical antimicrobial agents, the lysozyme has effective antibacterial activity to the drug resistance. For AMP and lysozyme, the difference in bactericidal mechanism leads to the different antibacterial spectrum. Thus, fusion expression of the two proteins may make up the antibacterial spectrum and enhance its stability. Thereby, it will play an important role in antimicrobial activity and provide new methods for treatment of drug-resistant infection.

Methylotrophic yeast expression system combines the advantages of both the prokaryotic expression system (characterized by high expression levels, easy scale-up, and inexpensive growth media) and the eukaryotic expression system (with the capacity to carry out many posttranslational modifications)[7]. Additionally, the methylotrophic yeast *Pichia pastoris* has emerged as a powerful and inexpensive heterologous expression system for the production of high levels of functionally active recombinant proteins[8] due to its use of the strong, tightly regulated methanol-inducible alcohol oxidase (AOX1) promoter[9]. If the new drug hLYZ-TP I fusion protein is developed and utilized to treat the infectious diseases of food source microbes, it will not only remove the current public health concerns over the antibiotic residues in human, but also provide a new strategy to curb the spread of drug resistance.

In this study, we report the functional expression and purification the hLYZ- TP I using *P. pastoris* expression system with signal sequence from pPICZαA vector to ensure production of properly folded secretory product. Meanwhile, the biological activity of purified hLYZ- TP I fusion protein was evaluated by minimum inhibitory concentration (MIC), Agar diffusion test (ADT) and Scanning electron microscope (SEM) analysis.

The cDNA fragment encoding the hLYZ-TP I gene was inserted to *P. pastoris* expression vector pPICZαA. And then transform the Sac I-linearized recombinatant plasmid into the yeast genome by electroporator. Acid washed glass pearls were used to extract the genomes of these 64 clones. PCR amplification results showed that all of them were positive clones. After flask expression, the protein was purified with the Ni 2+-sepharose affinity chromatography. The eluted fractions and the initial supernatant were analysed by SDS-PAGE, which demonstrated that the pure protein is what we want.

And then, we used the Minimum Inhibitory Concentration (MIC), Agar Diffusion Test (ADT) and Scanning Electron Microscope (SEM) to analyze the fusion protein's antimicrobial activity.

Firstly, micro-dilution method[10] was used to check the MIC for the fusion protein. In the frame of

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this research survey, various bacteria (Gram-positive and Gram-negative) were examined regarding their susceptibility against the fusion protein. More specifically, the bacteria *S. aurous* (ATCC25923), *S. aurous* (MRSA), *Streptococcus mutants* (ATCC55121), *B. subtilis* (CMCC63501), *Blastomyces albican* (ATCC10231), *L.monocytogenes* (CMCC54002) and *Bacillus cereus* (CMCC63301) were tested for Gram-positive while *S. typhi* (ATCC14028), *E. coli* (ATCC25922), and *Klebsiella pneumonia* (clinically isolated) for Gram-negative bacteria.

Next, test of antimicrobial activity of the fusion protein was conducted with the method of ADT[11] by using these strains as used above. All the results for the MIC and ADT are summarized in Table 1, Table 2.

The results demonstrated that as follows: firstly, compared to hLYZ and TP I, the inhibition of the fusion protein against S. aureus (ATCC25923), S. aureus (MRSA), Streptococcus mutans (ATCC55121), E. coli (ATCC25922), S. typhi (ATCC14028), and L.monocytogenes (CMCC54002) was attenuated; the antibacterial activity on *B. subtilis* (CMCC63501) was enhanced; and the effect on *Blastomyces albican* (ATCC10231) was invariant. Secondly, compared with TP I, the inhibition effects on *B. subtilis* (CMCC63501) was enhanced. Generally speaking, it can be found from these results that the antimicrobial activity of the fusion protein was weak, compared with the single hLYZ or TP I. However, it should be noted that the antibacterial spectrum of the new fusion protein was expanded through fusion expression. The pathogens, which were only sensitive to hLYZ or TP I, could be inhibited by the new fusion protein, such as *S. typhi*, *Klebsiella pneumonia*, *Bacillus cereus,* and *Blastomyces albican.* We can learn from the results of the *in vitro* inhibition that the fusion protein had the antibacterial activity of some gram-positive bacteria and gram-negative bacteria. However, compared with the natural human lysozyme and Tachyplesin I, the antibacterial activity of the recombinant fusion protein was not ideal. The biological activity in the fusion protein will be reduced under two conditions. Firstly, although the steric hindrance could be avoided by the linker, the helix-forming linker as well as the length of 12-mer, may make the two domains interfere with each other, which can lead to covering up the active regions. The length and sequence of the linker peptide could significantly affect the properties of the fusion protein. Several studies have focused on the effect of different lengths of linker peptides on the properties of single-chain Fv (ScFv) and fluorescence resonance energy transfer[12-13]. Not only was the composition of this linker important, but also its length[14]. Moreover, the human lysozyme and Tachyplesin I contain Cys', and the mismatching of Cys' was an indication of the interference of the two moieties with each other in the conformation of the fusion protein[15]. But, the

Table 1. MIC Values of the Fusion Protein against Different Kinds of Bacteria

Microorganism	Strain	MIC Values (μg/mL)
S. aurous	ATCC25923	25
S. aurous	MRSA	50
Streptococcus mutants	ATCC55121	25
Blastomyces albican	ATCC10231	50
B.subtilis	CMCC63501	12.5
S. typhi	ATCC14028	25
Klebsiella pneumonia	clinical isolated	25
E. coli	ATCC25922	25
L. monocytogenes	CMCC54002	50
Bacillus cereus	CMCC63301	25

Table 2. The Three Kinds of Eukaryotic Expression Products of a Variety of Bacteria Inhibitory Loop (unit: mm)

Microorganism	Strain	hLYZ	TP I	hLYZ-TP I
S. aurous	ATCC25923	19.0	22.0	15
S. aurous	MRSA	19.0	22.0	15
Streptococcus mutants	ATCC55121	15.0	14.0	13.5
Blastomyces albican	ATCC10231	13.0	-	13.0
B. subtilis	CMCC63501	16.0	22.0	26
S. typhi	ATCC14028	-	18.0	10
Klebsiella pneumonia	clinical isolated	-	18.0	17
E. coli	ATCC25922	17.0	26.0	16
L. monocytogenes	CMCC54002	17.0	26.0	14.0
Bacillus cereus	CMCC63301	-	18.0	16.5

fusion protein increased the inhibition against *S. typhi*, *Bacillus cereus* and *Klebsiella pneumoniae* compared to the human lysozyme. Compared with TPI, the inhibition of *Blastomyces albican* was increased. The anti-microbial spectrum of the fusion protein was expanded. The expanding of the anti-bacterial spectrum may be related with the changes about physical and chemical properties, which were showed in Table 3. These parameters were obtained by using ProtParam tool which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL or for a user entered sequence. It can be known from Table 3 that compared with the human lysozyme and Tachyplesin I, there are some significant differences in the theoretical pI, instability index and aliphatic index of the fusion protein. The solvent accessible surface (SAS), surface electrostatic potential distribution (SEPD) binding free energy and interaction residues are related to the interaction between the fusion protein and the bacteria[16].

At last, in order to make an in-depth study of the antimicrobial activity, *E. coli* (ATCC25922) and *S. aureus* (ATCC25923) were used to perform the SEM[17] analysis as the representatives of the Gram-negative and Gram-positive bacteria respectively.

The untreated *E. coli* cells, displayed a smooth and intact surface (Figure 1A). After incubation with hLYZ-TP I, the bacteria shortened and significantly increased their compactness in the SEM preparations (Figure 1C), indicating that *E. coli* was not able to grow to the maximum length. Moreover, leakage of cellular contents was observed in the preparations on silicon after incubation with the fusion protein (Figure 1E). In the control samples of *S. aureus*, the cells looked round and undamaged (Figure 1B). After incubation with the fusion protein, some bacteria had holes in their cell wall (Figure 1D). In addition, we found some bacteria had burst with deep craters in their cell wall (Figure 1F).

Table 3. The Physical and Chemical Properties of Human Lysozyme, Tachyplesins, and Human Lysozyme-tachyplesins

	Theoretical pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity (GRAVY)
Human lysozyme	9.14	34.08	69.85	-0.457
TP I	9.93	13.22	40.00	-0.518
hLYZ-TP I	9.36	36.25	61.89	-0.458

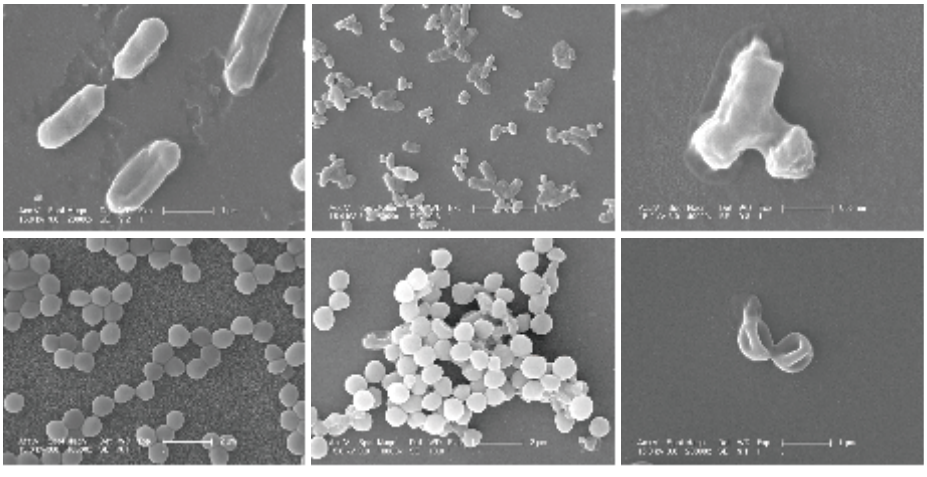

Figure 1. SEM micrographs of E. coli and S. aureus cells. The untreated E. coli cells are long and intact (A). After treatment with 5 times MIC (125 μg/mL) of the fusion protein, the cells look shorter (B) and visible leakage of cellular contents can be seen (C). The untreated S. aureus cells are round and intact (D). After treatment with 5 times MIC (125 μg/mL) of the fusion protein, some holes in the cells can be seen (E), and some completely lysed cells are found (F).

It was obviously indicated that the fusion protein can hydrolyze the β-1, 4 glycosidic bonds

between N-acetyl glucosamine and N-N-acetylmuramic acid of the Gram-positive bacteria cell wall and then the bacteria cell burst just because the changing of osmotic pressure. Meanwhile, the hLYZ-TP I also made hole on the bacteria surface. After that, bacteria died from the leakage of cellular content, which can be testified by the bacteria morphology what we found on the SEM after treated with hLYZ-TP I. SEM analysis suggested that the "double-function" of the fusion protein appeared on killing bacteria, which revealed that the "dual mechanism" for antibacterial can be created by this design. We can make a hypothesis that when the fusion protein acts on killing bacterial, firstly, the hLYZ hydrolyzes β-1, 4 glycosidic bonds, damages the cell wall and then TP I punches on the surface easily, which called "cooperation".

Based on the human lysozyme genes cloned from human placenta tissue and synthetic antibacterial peptide Tachyplesin I genes, hLYZ- TP I fusion protein was expressed in the eukaryotic expression system and had high biological activity. The analysis and comparison of expression products showed that it was essential to optimize the linker peptide to achieve higher biological activity recombinant fusion protein, which is called "Additive Effect". This provided a new idea and method for prevention and treatment of clinical diseases.

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