Short Communication

Bactericidal Action of Lysozyme against Gram-negative Bacteria Due to Insertion of a Hydrophobic Pentapeptide into Its C-Terminus

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Lytic activity of hen egg-white lysozyme is limited to certain Gram-positive bacteria, but it does not act on Gram-negative bacteria including foodborne pathogens. Thus, any attempts to convert lysozyme to be active in killing Gram-negative bacteria would be an important contribution for modern biotechnology and medicine. The cell envelope of Gram-negative bacteria is a complex structure composed of three morphologically distinct layers, a cytoplasmic membrane, a rigid peptidoglycan layer external to the cytoplasmic membrane, and the outer membrane at the surface of the cell. Access of lysozyme to the peptidoglycan layer of the cell wall to perform its action is hindered by the outer membrane permeability barrier. Therefore, in order for lysozyme to bind and fuse into the outer membrane, we postulated that it may be converted into a membrane-penetrating form if equipped with a hydrophobic carrier. In this respect we have recently succeeded in developing a lysozyme with significant antimicrobial action against Gram-negative bacteria by covalent attachment of one or two palmitic acid residues to lysyl residues of the molecule.1) This rational result has invited an approach that we followed in this study. Here, our strategy of designing an effective antimiicrobial agent for Gram-negative bacteria is based on the insertion of a hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro), which can assume an extended β-strand configuration and thus provide the same length as a palmitoyl residue, into the C-terminus of lysozyme to enable it to fuse into the outer membrane, a fusogenic protein-mimetic approach. Fusogenic proteins are proteins that can pass through biological membranes from the extracellular side, such as colicins, mellitin, α-toxin, and viral hemagglutinin HA.2-4) This paper reports that fusion lysozyme is a potent bactericidal molecule against Gram-negative bacteria (Escherichia coli as a representative microorganism) in both neutral and acidic pH.

The DNA sequence of lysozyme suggested an obvious way of fusing the hydrophobic peptide immediately after its C-terminus. The construction of the fusion gene of lysozyme is shown in Fig. 1. Lysozyme cDNA was cloned in an M13 vector as described previously.5) To introduce an appropriate site (PstI) for insertion of the fusion peptide, oligonucleotide-directed mutagenesis was employed by which the base C 465 of Arg 128 codon was converted to A. This single base substitution had no effect on the coded amino acid (Arg 128). The resulting plasmid was digested with PstI endonuclease, followed by ligation with a chemically synthesized 28-mer double stranded linker, encoding the codons of Arg 128 and Leu 129 of the lysozyme C-terminus to compensate for those deleted upon digestion with PstI, five codons of the fusion hydrophobic peptide (FFVAP), and the stop codon. Plasmids carrying the correctly inserted sequence were identified by DNA sequencing using a synthetic primer oligodeoxynucleotide (5'-GGCAGGACCCCAGCTCAGG-3') complementary to the coding sequence from Gly 67 to Arg 73 of hen egg-white lysozyme cDNA. The full-length cDNA of either wild-type or fusion lysozyme was isolated from an M13 clone by double digestion with EcoRI and HindIII, and blunt-ended. The DNA fragment was then fused with the SalI site downstream of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter of the yeast expression vector pYG-100, as reported earlier.5) These expression plasmids were introduced into Saccharomyces cerevisiae

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Figure 1. Scheme for Construction of Fusion Gene of Lysozyme.

We first generated a PstI site in the Cys127 and Arg128 codons by site-directed mutagenesis with which the base C465 was converted to A (bold-typed nucleotide). Two synthetic DNA fragments, encoding the hydrophobic sequence (italic style), were inserted into the mutant gene upon digestion with PstI to construct fusion M13 clone, pHLL-5. The codons for amino acids deleted upon digestion with PstI were compensated on the 5'-end of the inserted DNA linker. The expression plasmids of wild-type and fusion lysozyme were constructed by inserting EcoRI-HindIII fragment (full-length cDNA) of pPLL465 and pHLL-5 into SalI site of a yeast expression vector, pYG-100, respectively. In cDNA: black box, signal peptide sequence; striped box, coding region of hen egg white lysozyme; open box, 3'-noncoding region. The plasmids are not drawn to the scale.
AH22 (a, leu 2, his 4, cir^2) by the lithium acetate procedure of Ito et al. Transformants harboring the recombinant plasmid were screened on Leu^- minimal medium plates. Yeast cells carrying the recombinant plasmids were grown in yeast minimal medium at 30°C for 5 days, as previously described. The fusion lysozyme (H5-Lz) secreted in the culture medium as well as wild-type protein were purified by two steps of cation-exchange chromatography on CM-Toyopearl. The active protein peak was collected and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) or sodium acetate buffer (pH 5.0). It was estimated that the yield of H5-Lz was approximately 60—70% that of wild-type protein. This agrees with a recent report that fusion of a hydrophobic stretch of five leucine residues had no drastic effect on the translocation and secretion of interleukin 2. It should be noted that lytic activity of the fusion lysozyme against Micrococcus lysodeikticus was 80% of wild-type protein when measured in 100 mM sodium phosphate buffer (pH 6.0). This suggested that the fusion of the hydrophobic pentapeptide had undramatic effect on the enzymatic activity of lysozyme. Therefore, this residual activity is sufficient to cause lysis of the inner membrane upon penetration into the outer membrane of Gram-negative bacteria.

Antibacterial action of fusion lysozyme was examined against E. coli wild-type 3301, as a representative strain of Gram-negative bacteria. In certain experiments, a suspension of E. coli cells (10^5 cells/ml) of mid-logarithmic phase culture, was mixed with various protein concentrations of lysozymes in 10 mM sodium phosphate buffer (pH 7.0) or sodium acetate buffer (pH 5.0). The mixture was incubated at 37°C for 10 min. A 100-μl portion or dilution was plated onto two separate nutrient agar plates (MacConkey). Viable cells were counted after incubation at 37°C for 24 hr. Controls contained bacteria incubated in buffer alone. Percentage survival was represented with respect to the control mixture. Figure 2 shows the antibacterial actions of authentic, wild-type, and fusion lysozymes against E. coli WT-3301 as a function of protein concentration at pH 7.0. As shown, when E. coli suspensions were incubated with 2.5 μg protein, the authentic and wild-type lysozymes had undetectable bactericidal action. By contrast, fusion lysozyme H5-Lz exhibited significant antibacterial action, where more than 50% of E. coli population was killed. Further increase in the protein concentration of authentic and wild-type proteins accompanied with a slight decrease in viable E. coli number up to 7.5 μg protein, where it reached constant. On the other hand, H5-Lz manifested an additional linear decrease in E. coli viability up to the highest protein concentration examined (10 μg/ml). These results indicate that the fusion lysozyme elaborated in this study was proved to be a potent inhibitor of E. coli, as a model for Gram-negative bacteria, since it exerted substantial antibacterial action at protein concentration as low as 2.5 μg/ml (1.74 × 10^4 μm) and under physiological pH. Since low pH is known to activate membrane insertion and translocation of many fusogenic protein toxins, such as viral hemagglutinin, colicins and various amphipathic toxins, we speculated that testing the bactericidal action of fusion lysozyme at pH 5.0 should provide insight into possible similarity between H5-Lz and the above mentioned proteins in terms of membrane insertion process. Table I illustrate the bactericidal action of wild-type and fusion lysozymes at neutral (pH 7.0) and acidic (pH 5.0) incubation conditions of E. coli suspension 10^8 cells/ml with 1.74 × 10^4 μm protein. Nearly 90% of the E. coli population survived in the presence of wild-type protein at either neutral or acidic pH conditions. As expected, lowering the pH to 5.0 greatly enhanced the bactericidal action of fusion lysozyme, where it severely reduced the colony forming capability of the exposed bacteria within 10 min. It seems likely that at low pH the fused hydrophobic sequence at the COOH-terminal of H5-Lz became exposed, and hence induced the binding and insertion of the H5-Lz molecule to the bacterial outer membrane and thus performed it action on the peptidoglycan layer. Particularly, the fused peptide is predicted to assume the β-strand configuration, which is proposed to reside within the lipid bilayer when protein penetrates the membrane.

A space-filling model suggested that the length of a single residue of palmitic acid is exactly equal to the length of the fused hydrophobic pentapeptide as predicted to adopt the β-strand configuration. Thus, our rational design of an antibacterial protein to Gram-negative bacteria from lysozyme by genetic manipulation of its amphiphilicity without use of chemical modification is confirmed. In addition, this approach suggests that it may offer a novel

![Fig. 2. Bactericidal Action of Wild-type and Fusion Lysozymes against E. coli WT3301 as a Function of Protein Concentration.](image)

The purified authentic (open square), wild-type (open circle), or fusion (solid circle) lysozymes were tested against E. coli (10^5 cells/ml) of mid-log phase culture, in 10 mM Na-phosphate buffer, pH 7.0. The proteins were tested at concentrations indicated at 37°C for 10 min. The results are mean values from three separate experiments and the bar indicates the standard deviation.

| Table I. Bactericidal Action of Wild-type and Fusion Lysozymes at Neutral and Acidic pH |
|-------------------------------|---|-------------------|
| Protein | pH  | % Survival of E. coli |
| WT-Lz | 7.0 | 89.9±3.2 |
|        | 5.0 | 87.4±2.6 |
| H5-Lz | 7.0 | 49.5±1.5 |
|        | 5.0 | 14.8±2.2 |

Proteins were tested at 1.74 × 10^4 μm (2.5 μg/ml) in 10^5 CFU/ml of E. coli WT-3301 suspension, mid-logarithmic phase culture, in 10 mM sodium phosphate buffer (pH 7.0) or 10 mM sodium acetate buffer (pH 5.0). The data are expressed as % survival with respect to controls (buffer alone). Each experiment consisted of triplicate determinations.
Hydrophobic Pentapeptide-fused Lysozyme

mean of inhibiting Gram-negative bacteria with a safe protein which has long been used as an anti-inflammatory drug. Further study to elucidate the conformational changes associated with the hydrophobic fusion as well as the mechanism of bactericidal action is now in progress.

References