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Bactericidal action of lysozymes attached with various sizes of hydrophobic peptides to the C-terminal using genetic modification

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Abstract The genetic modification of lysozyme was attempted to improve the bactericidal activity against Gram-negative bacteria *E. coli*. The different lengths of hydrophobic peptides were attached to the C-terminus of the hen egg white lysozyme to investigate the most effective length of the hydrophobic peptides for killing bacteria. The oligonucleotides encoding Phe-Val-Pro (H3), Phe-Phe-Val-Ala-Pro (H5) and Phe-Phe-Val-Ala-Ile-Ile-Pro (H7) were fused to the C-terminus Leu 129 of lysozyme cDNA. The reconstructed cDNAs were inserted into the yeast expression vector. The hydrophobic peptide-fused lysozymes were secreted in the yeast carrying the reconstructed cDNA. Although the hydrophobic peptide-fused lysozymes retained 75–80% lytic activity of the wild-type protein, the bactericidal action to *E. coli* was greatly increased with the length of hydrophobic peptides. These results suggest that the hydrophobic peptides play an important role in killing Gram-negative bacteria. To elucidate the role of catalytic domain in bactericidal action of the hydrophobic fusion lysozyme (H5-Lz), the mutant hydrophobic lysozyme (H5/E35A-Lz) whose glutamic acid was substituted with alanine at the position 35 was constructed to diminish the catalytic activity. The mutant hydrophobic lysozyme (H5/E35A-Lz) was greatly lost the bactericidal action to *E. coli*, suggesting that not only the length of hydrophobic peptide fused to C-terminus but also the catalytic domain is important for the bactericidal action of the hydrophobic peptide-fused lysozyme.

Key words: Hydrophobic peptide-attached lysozyme; Secretion of lysozyme in yeast; Enhanced bactericidal action of lysozyme

1. Introduction

Lysozyme is one of the most popular and safe bactericidal proteins. However, the antimicrobial action of lysozymes is limited to part of the Gram-positive bacteria. Therefore, it is desirable to broaden the antimicrobial specificity to Gram-negative bacteria by designing newly lysozyme. The differences in the bactericidal action between Gram-positive and Gram-negative bacteria are due to the cell envelope of the latter, which consists of the outer membrane, inner membrane and peptidoglycan layer. Thus, the enhancement of lysozyme penetration through the membrane barrier was attempted using chemical [1] and genetic [2] modifications. The fatty acid binding and pentapeptide fusion into the C-terminus are very effective to kill the typical Gram-negative bacteria *E. coli*. These findings suggest that such hydrophobic domains may promote the penetration of the lysozyme molecule into the inner membrane and the subsequent loss of the electrochemical membrane potential [2]. However, it remains to be solved how long hydrophobic peptide fused to the C-terminal of lysozyme is optimal for the antimicrobial activity and whether the catalytic domain is essential for the bactericidal action to Gram-negative bacteria or not. In this paper, we intended to elucidate the effects of the length of hydrophobic peptide attached to C-terminus in lysozyme on the antimicrobial action against *E. coli* using genetic modification of the lysozyme. In addition, the role of the catalytic domain in the bactericidal action was also investigated by using hydrophobic lysozyme mutated at the catalytic site. The secretion of lysozyme inserting hydrophobic domains to its C-terminus was carried out in a yeast expression system where mature proteins are correctly processed. According to the quality control in endoplasmic reticulum in yeast, the secretion amounts of hydrophobic lysozymes are very small. To overcome this problem, we devised a method to obtain a considerable amount of mutant lysozymes. Thus, it has become feasible to further investigate the mechanism of the bactericidal action of hydrophobic peptide-fused lysozyme.

2. Materials and methods

2.1. Materials

The microbial substrate of a lysozyme, *Micrococcus lysodeikicus* cells, and LPS from *E. coli* K12 IFO3301 were purchased from Sigma. Synthesized oligodeoxyribonucleotides of mismatching primer for mutagenesis (5′-AGAGGCTGCAGGCTGTGA-3′) and the six DNA fragments encoding the fusion sequence synthesized by the phosphoamidate method were supplied by Takara Shuzo, Japan. Unless otherwise stated, all other chemicals were of the highest grade commercially available.

2.2. Bacterial strains and plasmids

E. coli K12 TG1 (Δ(*lac-pro*)*supE, thi, hsd, D5/F′ tra D36, proA*$^+$*B*$^+$, *lacIq, lacZ DM15*) used as a host cell and M13 mp19 bacteriophage were supplied by Amersham Japan and Takara Shuzo, respectively. *E. coli* K12 IFO 3301 (*lacI lacO lacZ lacY*) was used primarily as a representative microorganism for Gram-negative bacteria. The pYG100 [3], an *E. coli* yeast shuttle vector, was provided by Dr. K. Matsubara, Osaka University. *Saccharomyces cerevisiae* AH22 (*a, Leu2, his4, Cir*$^+$), an expression strain, pKK1 plasmid [4], which contains the full length of the prelysozyme cDNA in the same orientation as lacZ′ in pUC18, and pYGKK-1 [4] that was constructed by inserting the blunt-ended, full-length cDNA into the *Sal*I site of pYG100, were provided by Dr. I. Kumagai, University of Tokyo.

2.3. Construction of expression plasmids of hydrophobic peptide-fused lysozymes

A *Pst*I site was introduced into the 3′-end of the coding region of the prelysozyme cDNA by the oligonucleotide-directed mutagenesis using a synthesized primer, 5′-AGAGGCTGCAGGCTGTGA-3′, as previously reported [2]. The resulting plasmid, designated pPLz465, was digested with *Pst*I, followed by ligation with each two complementary synthesized oligonucleotide fragments (5′-GGCTGTTTGT-CCCCTGAC-TGCA-3′ and 5′-ACGTCCGACAAACAGGGGAC-TG-3′ for H3, 5′-GGCTGTTT-TTTGTCGCTCCCTGACTGCA-3′ and 5′-ACGTCCGACAAAAAACAGCGAGGGA-CTG-3′ for H5,

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5′-GGCTGTTTTTTGTCGCTATCATCCCCTGACTGCA-3′ and 5′-ACGTCCGACAAAAAACAGCGATAGTAGGGGACTG-3′ for H7), which contain the codons of Arg128 and Leu129 of lysozyme gene, each fusion hydrophobic sequence (H3, Phe-Val-Pro; H5, Phe-Phe-Val-Ala-Pro; H7, Phe-Phe-Val-Ala-Ile-Ile-Pro), and stop codon, respectively. The fusion plasmid was corrected and confirmed by Southern hybridization and DNA sequencing. The Southern hybridization of bacteriophage plaques was essentially carried out. These fusion plasmids are termed pHLz-3, pHLz-5, pHLz-7, respectively. The small *Mlu*I-*Sal*I fragment of the fusion cDNA was isolated from pHLz-3, 5 and 7, respectively, and ligated with the large *Mlu*I-*Sal*I fragment of the pYGkk-1 plasmid to construct the expression plasmid. These expression plasmids are termed pYGHL-3, pYGHL-5 and pYGHL-7, respectively.

The mutant lysozyme cDNA (G49N) having an N-glycosylation site at position 49 was constructed by site-directed mutagenesis and inserted into the *Sal*I site of pYG100 (G49N pYGkk-1) [4]. To construct the plasmid of the N-glycosylated fusion lysozyme, the small *Mlu*I-*Sal*I fragment was isolated from the fusion cDNA and ligated with the large *Mlu*I-*Sal*I fragment of the G49N pYGkk-1 plasmid. These expression plasmids of the N-glycosylated fusion lysozyme were termed H3/G49N, H5/G49N and H7/G49NpYGkk-1.

The mutant hydrophobic lysozyme cDNA (H5/E35A) whose glutamic acid was substituted with alanine at the position 35 was constructed by site-directed mutagenesis. The mutagenic oligonucleotide primer, 5′-GTTGAAGTTACTAGCGAATTTTGCG-3′ was used for PCR in vitro mutagenesis kit (Takara, Japan). The mutant cDNA was inserted into the *Sal*I site of pYG100.

2.4. Expression of hydrophobic peptide-fused lysozymes

The yeast expression plasmids of mutant HEWL were introduced into *S. cerevisiae* AH22 according to the lithium acetate procedure [5]. The leu$^+$ transformants were selected on a yeast minimal medium (YMM) plate supplemented with 20 μg/ml of histidine at 30°C. The transformant carrying the pYGkk-1 of mutant HEWL was inoculated into 3 ml of YMM and incubated for 2 days at 30°C with shaking. This preculture was subcultured to 100 ml of the same medium in a flask (500 ml) and incubated another 2 days at 30°C with shaking. Ten ml of the second preculture was then transferred to 1 l of YMM in a 3 l flask and cultured under the same conditions.

2.5. Purification of hydrophobic peptide-fused lysozymes

The growth medium of *S. cerevisiae* AH22 described above was centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was applied to a CM-Toyopearl 650 column (1.5×5.0 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5), and after washing the column, protein was eluted with the same buffer containing 0.5 M NaCl. Fractions containing the lysozyme were pooled, diluted 10 times with the same buffer and rechromatographed on a CM-Tyopeal 650 column (1.5×10 cm). The lysozyme was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. The fractions having enzymatic activity were collected and dialyzed in a Spectra/por dialysis bag (M_r 3500 Da cut off) against deionized water.

2.6. Lytic activity of hydrophobic peptide-fused lysozymes

The lytic activity of the fusion lysozyme derivatives against *M. lysodeikicus* cells was determined according to a turbidometric method [6] based on the decrease in turbidity of a cell suspension with the addition of fusion lysozyme derivatives. A *M. lysodeikicus* cell suspension (170 μg/ml) was prepared as a substrate in 50 mM sodium acetate or sodium phosphate buffer was monitored at 450 nm. A 100 μl portion of the lysozyme derivatives solution (37 μg/ml) was added into 2.4 ml of substrate and the decrease in the absorbance at 450 nm for 1 min using a Hitachi U-2000 spectrophotometer was monitored. The K_m value was determined by the hydrolytic activity of ethylene glycol chitin using the reducing procedure [7]. The 0.01 to 0.1% ethylene glycol chitin solutions (10 mM acetate buffer, pH 5.5) were incubated with 10 μg lysozymes at 40°C for 30 min. After the reaction, 2 ml of color reagent (made by dissolving 0.5 g potassium ferricyanide in 1 l of 0.5 M sodium carbonate) was added, and the mixture was immediately boiled for 15 min to estimate the reducing power resulting from hydrolysis of ethylene glycol chitin.

The K_m values were calculated from reciprocal plot of the reaction rate against the concentration of substrate.

SDS-polyacrylamide gel electrophoresis was performed using a 5% acrylamide stacking gel and 15% of the separating gel containing 0.1% SDS according to the method of Laemmli [8]. After electrophoresis, the gel sheets were stained for protein and carbohydrate with 0.2% Coomassie Brilliant blue R250 and with 0.5% periodic acid and Fuchsin-basic solutions [9], respectively.

2.7. Solid phase ELISA

The wells of microtiter plates were seeded with 100 μl of the antigen (100 μg/ml) in carbonate buffer and coated with 1% Bovin serum albumin in PBS(-). The plates were rinsed four times with PBS(-) containing 0.05% Tween20 (PBS(-)Tween), and 100 ml of mAbs (100 mg/ml) in PBS(-) were added to each well. After the plates were washed with PBS(-)Tween, 100 ml diluted goat anti-mouse antibody conjugated with peroxidase (10^3 times) was added to each well. After washing with PBS(-)Tween, 100 ml of the substrate (0.04% *o*-phenylenediamine and 0.02% H_2O_2 in 0.05 M citrate-0.1 M phosphate buffer at pH 5.0) was added. The reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4, and the absorbance of each well was measured at 490 nm on a microplate reader (BIO-RAD Model 450).

2.8. Antimicrobial assay

The measurement of the bactericidal action of the fusion lysozyme derivatives against Gram-negative bacteria was carried out using *E. coli* K-12 IFO 3301 as a representative strain. The suspension of *E. coli* cells (10^5 cells/ml) from the mid-logarithmic phase culture was mixed with various concentrations of lysozymes in 50 mM sodium acetate buffer (pH 5.5). The mixture was incubated at 37°C for various lengths of time. At a given time, 100 μl portions or dilutions were plated onto two separate nutrient agar plates (MacConky). All plates were incubated at 37°C overnight (~14 h), and then the viable cell numbers were counted. Percent survival was based on the colony number to a control solution without lysozyme.

2.9. LPS binding assay

Lytic action of lysozyme types against *M. lysodeikticus* cells was monitored in the presence of purified LPS (lipopolysaccharide from *E. coli*) essentially as above except that the concentration of LPS in buffer was varied against a single concentration of lysozymes (final concentration, 1 μg/ml). The mixtures were incubated at 37°C for 15 min before adding the *M. lysodeikticus* cells suspension. The final volume of the lysis mixture was always kept at 2 ml. The residual activity is expressed as a percentage of that observed in the absence of LPS.

3. Results

3.1. Expression and purification of hydrophobic peptide-fused lysozymes

A significant amount of wild-type lysozyme was secreted into the medium of *S. cerevisiae* cells harboring the expression plasmid (pYGKK-1), while the tripeptide-fused lysozyme (H3-Lz) and pentapeptide-fused lysozyme (H5-Lz) were slightly secreted, and the heptapeptide-fused lysozyme (H7-Lz) was not secreted. In order to increase their secretion, the glycosylation of the fusion lysozyme was intended to protect the mutant protein from proteolysis in yeast cells. The hydrophobic peptide-fused lysozymes with the recognition sequence (Asn-X-Thr/Ser) site at position 49 to attach the N-linked oligosaccharide chain (H3/G49N-Lz, H5/G49N-Lz, H7/G49N-Lz) were significantly secreted into the medium, although the amounts of secretion were lower than that of

Table 1
K_m values of hydrophobic peptide-fused lysozymes

Lysozyme	K_m (mg/ml)
Wild	0.0156 ± 0.008
Tripeptide-fused	0.0220 ± 0.012
Pentapeptide-fused	0.0232 ± 0.012

Values are the means from four independent experiments. The standard deviations are shown.

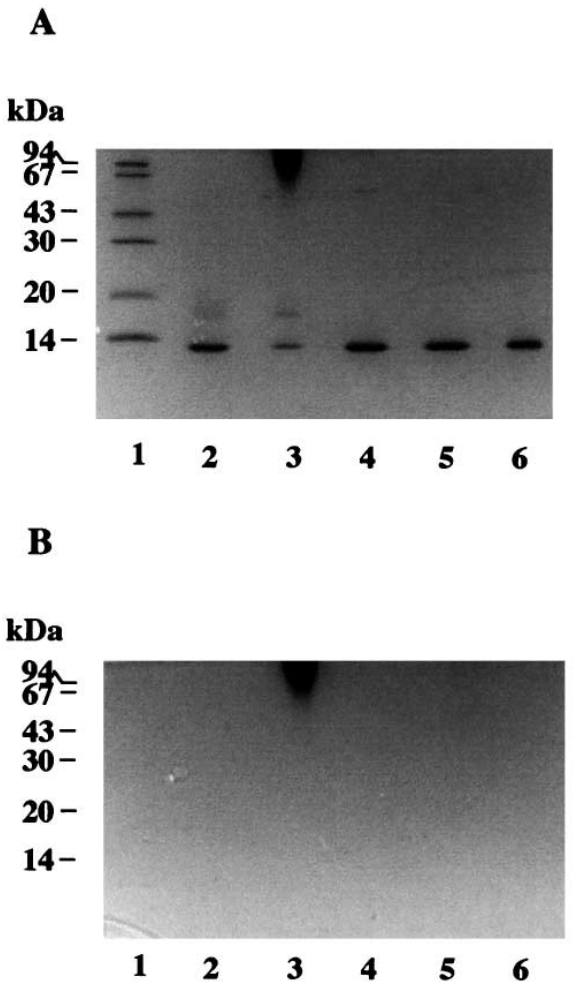

Fig. 1. SDS-PAGE patterns of hydrophobic peptide-fused lysozymes secreted from *S. cerevisiae*. The gel sheets were stained for protein and carbohydrate with Coomassie Brilliant Blue (pamel A) and periodic acid-Fuchsin (panel B), respectively. Lane 1, molecular weight markers (94000, phosphorylase b; 67000, bovine serum albumin; 43000, ovalbumin; 30000, carbonic anhydrase; 20100, trypsin inhibitor; 14300, α-lactalbumin); lane 2, wild-type-Lz; lane 3, G49N-Lz; lane 4, H3/G49N-Lz; lane 5, H5/G49N-Lz; lane 6, H7/G49N-Lz.

the wild-type lysozyme. The secreted amounts of hydrophobic peptide-fused lysozymes were about 50 μg/l. The SDS-PAGE patterns of H3/G49N-Lz, H5/G49N-Lz and H7/G49N-Lz were different from that of G49N-Lz. The G49N-Lz was secreted mostly in the polymannosyl form, but the hydrophobic peptide-fused lysozymes were secreted in the non-glycosyl forms of the protein (Fig. 1). This is because the hydrophobic domains may affect the folding of the fusion lysozymes, thereby resulting in the inhibition of the glycosylation process in the endoplasmic reticulum. Thus, the non-glycosylated lysozymes were secreted in the medium. This is convenient for investigating the effect of the size of hydrophobic peptide fused to the C-terminus of the lysozyme on the bactericidal action. Since the lysozyme secreted in the medium was reported to be purified by two steps of cation-exchange chromatography on a CM-Toyopearl column [4], the hydrophobic peptide-fused lysozymes were also purified by the same procedure. As shown in Fig. 1, the SDS-PAGE patterns of the hydrophobic tripeptide- (lane 4), pentapeptide- (lane 5) and

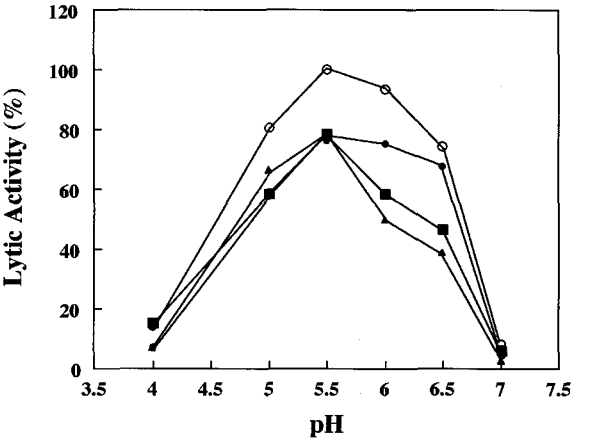

Fig. 2. Lytic activities of various lysozymes as a function of pH values. The decrease in turbidity at 450 nm of *M. lysodeikticus* cell suspension in 600 μl of 50 mM buffer was determined at different pH values. The lysis was monitored in the presence of 3.7 μg/ml. Wild-Lz (○), H3/G49N-Lz (●), H5/G49N-Lz (■), H7/G49N-Lz (▲). The activity is expressed as a percentage of that observed for wild-Lz at pH 5.5.

heptapeptide- (lane 6) fused lysozymes showed to be a completely purified form. These lysozymes were used in the experiments.

3.2. Enzymatic activity of hydrophobic peptide-fused lysozymes

The enzymatic activity of the hydrophobic peptide-fused lysozymes were measured from the degree of lysis of *M. lysodeikticus*. The lytic activities of the fusion lysozymes were about 75–80% of that of the wild-type lysozyme below pH 5.5, while it greatly decreased above pH 6, especially for pentapeptide- and heptapeptide-fused lysozymes (Fig. 2). This suggests that the effect of hydrophobic peptide fusion on the conformation of lysozyme is smaller at low pH than above pH 6. The optimal pHs of these fusion lysozymes were 5.5 as well as that of the wild-type lysozyme. Table 1 shows the apparent

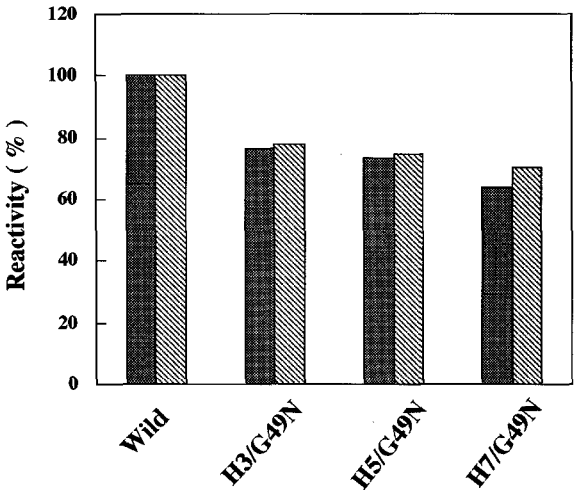

Fig. 3. Binding of mAb 4G5 (left-side dark blocks) and TL G1100 (right-side dashed blocks) with hydrophobic peptide-fused lysozyme. The binding reactivity of mAb with hydrophobic peptide-fused lysozymes was expressed as a percentage of that observed for wild lysozyme. The standard deviations of five replicates were between 2.5 and 7.0% in all samples.

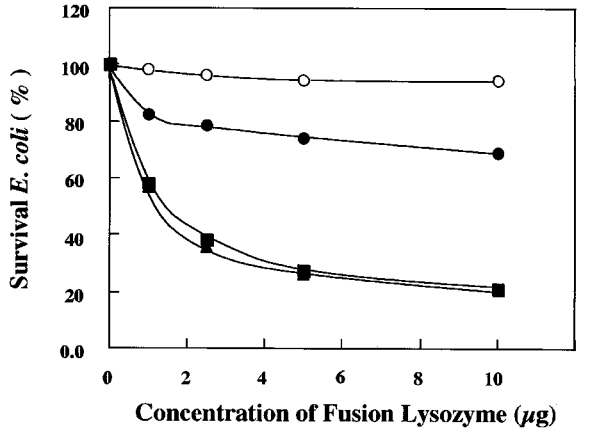

Fig. 4. Bactericidal action of wild and hydrophobic peptide-fused lysozymes to *E. coli* cells. The different concentrations of wild-Lz (○), H3/G49N-Lz (●), H5/G49N-Lz (■), H7/G49N-Lz (▲) were incubated with the *E. coli* cells (10^5 cells/ml) at 37°C for 30 min in 50 mM sodium acetate buffer, pH 5.5.

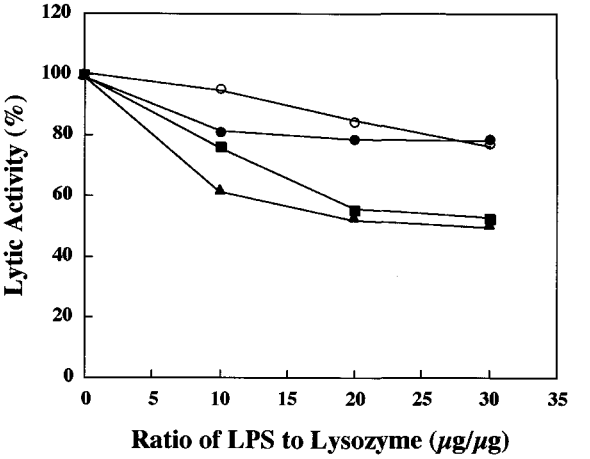

Fig. 5. Lytic activities of various Lz at pH 5.5 in the presence of LPS. One μg/ml of wild-Lz (○), H3/G49N-Lz (●), H5/G49N-Lz (■), H7/G49N-Lz (▲) were incubated with different concentrations of the *E. coli* LPS at 37°C for 15 min in sodium acetate buffer, pH 5.5. The solution (0.1 ml) was mixed with 1.9 ml of *M. lysodeikticus* cell suspensions in the same buffer and the decrease in the absorbance at 450 nm was monitored. The activity is expressed as a percentage of that observed in the absence of LPS.

K_m values calculated from the hydrolytic activity of glycol chitin. The K_m values of hydrophobic peptide-fused lysozymes were slightly larger than that of wild lysozyme, suggesting the lower of affinity to substrate. These results suggest that the conformation of lysozyme is slightly strained by the fusion of hydrophobic peptides to C-terminus without dramatic loss of lytic activity. The two monoclonal antibodies (mAbs), 4G5 and TL G1100, sensitive to the conformational change in the lysozyme [10] were used for the conformational analysis of the hydrophobic peptide-fused lysozymes. Fig. 3 shows the binding reactivity of mAb 4G5 and TL G1100 with the hydrophobic peptide-fused lysozyme in the solid phase ELISA. The reactivity of the hydrophobic peptide-fused lysozymes, H3/G49N-Lz, H5/G49N-Lz and H7/G49N-Lz, were 76.9%, 73.5% and 63.6%, respectively (against 4G5) and 78.2%, 74.9% and 70.1%, respectively (against TL G1100). These results indicate the fusion of the hydrophobic peptides to lysozymes causes the conformational change in proportion to the length of the peptides. Despite the conformational changes, the lytic activity may be conserved enough to kill bacteria. Therefore, it was expected that the antimicrobial activity of hydrophobic fusion lysozyme was extended to Gram-negative bacteria to which wild-type lysozyme was insensitive.

3.3. Antimicrobial action against *E. coli* of hydrophobic peptide-fused lysozyme

The effect of the length of hydrophobic peptides in fusion lysozymes on the antimicrobial activity against *E. coli* was estimated at pH 5.5. As shown in Fig. 4, the wild-type lysozyme exhibited the value of *E. coli* survival of 94.2%, while H3/G49N-Lz, H5/G49N-Lz and H7/G49N-Lz exhibited a value of 69.2, 21.3 and 20.8%, respectively, in a dose-dependent manner. It is interesting that H5/G49N-Lz and H7/G49N-Lz showed almost the same antimicrobial action. This result indicates that the antimicrobial action increases with the length of the hydrophobic peptide fusion up to the pentapeptide. Thus, the potent antimicrobial activity to Gram-negative bacteria was endowed by the attachment of hydrophobic peptides to the C-terminus of lysozyme. However, it is not clear whether the lytic activity of the lysozyme is essential for the antimicrobial action or not, although the hydrophobic extension to the C-terminus of the lysozyme is critical for the antimicrobial activity. To elucidate this point, we constructed mutant hydrophobic peptide-fusion lysozyme whose glutamic acid was substituted with alanine at the position 35 to diminish the lytic activity. The roles of catalytically active residue glutamic acid-35 in lysozyme have been investigated by a site-directed mutagenesis [11]. The substitution of glutamic acid-35 with alanine exhibited the complete loss of the lytic activity. As shown in Table 2, the mutant pentapeptide-fused lysozyme (H5/E35A-Lz) without lytic activity greatly decreased the antimicrobial activity against *E. coli*, although the effect of the hydrophobic stretch slightly appeared. This result suggests

Table 2
Effects of the deletion of catalytic site Glu-35 on the bactericidal action and lytic activity of pentapeptide-fused lysozyme

Lysozyme	Survival *E. coli*[b]	Lytic activity[c]
Wild	94.7 ± 1.5	100
Pentapeptide-fused control	20.5 ± 0.9	77.6 ± 1.1
Mutant (H5/E35A)[a]	81.7 ± 1.4	0

Values are the means from four independent experiments. The standard deviations are shown.
[a]The hydrophobic fusion lysozyme (H5/G49N-Lz) was mutated at the position 35 whose glutamic acid is substituted with alanine to diminish the lytic activity.
[b]The suspension of *E. coli* cells (10^5 cells/ml) was mixed with 10 μg lysozymes at 37°C for 30 min in 50 mM sodium acetate buffer (pH 5.0). The values are represented as percent survival based on the colony number to a control solution without lysozyme.
[c]The decrease of turbidity at 450 nm for 1 min of *M. lysodeikticus* cell suspension in 50 mM sodium acetate buffer (pH 5.5) was monitored in the presence of lysozymes (3.7 μg). The lytic activity was represented as a percentage of that observed for wild-type lysozyme at pH 5.5.

that the catalytic domain is important for the antimicrobial action in combination with the hydrophobic domain.

3.4. Interaction between LPS from E. coli and hydrophobic peptide-fused lysozymes

The interaction of fusion lysozymes with LPS, the most abundant components in the outer membrane of Gram-negative bacteria, was investigated by monitoring the reduction of the lytic activity of lysozyme for *M. lysodeikticus* after incubation with various concentrations of LPS [12]. The incubation with LPS reduced the activity of the wild-type and fusion lysozymes in a dose-dependent manner. The lytic activity of the wild-Lz decreased with an increase in the LPS dose, while the activity of H3/G49N-Lz, H5/G49N-Lz and H7/G49N-Lz showed a sharp decrease to about 50% (Fig. 5). This indicates that the interaction of the hydrophobic peptide-fused lysozyme with LPS is strengthened with the increases in the length of hydrophobic tail.

4. Discussion

The hydrophobic peptide-fused lysozymes were secreted in enough amounts to characterize the antimicrobial and enzymatic properties by the substitution of glycine at position 49 with asparagine. The hydrophobic peptide-fusion lysozymes exhibited strong antimicrobial activity against *E. coli*. The effect of antimicrobial activity against *E. coli* of the hydrophobic fusion lysozyme has been ascribed to the hydrophobic peptide attached to the C-terminus, which may contribute to the penetration into the outer membrane of the bacteria. The optimal length of the hydrophobic residues was found to be more than five hydrophobic residue to strongly affect on the antimicrobial activity. The hydrophobic residues attached to the C-terminus are liable to form β-strand according to the secondary structure prediction [13]. The length of β-strand of pentapeptide corresponds to that of aliphatic side-chain in palmitic acid. When the hydrophobic residue forms the β-strand conformation, the length of the pentapeptide is long enough to penetrate into the lipid bilayer. Thus, it became apparent that the hydrophobic tail is closely involved in the killing action of *E. coli*. In addition, the importance of the catalytic domain of lysozyme was also confirmed for the antimicrobial action of hydrophobic peptide-fused lysozymes. The interaction between the LPS of the outer membrane of *E. coli* and the lysozyme increased with the length of the attached hydrophobic peptide. This interaction may be accentuated by electrostatic interactions between the abundant positively charged amino acid residues on the surface of the lysozyme molecule and negatively charged heads of the phospholipids or LPS. As a consequence, the rest of the lysozyme molecule transverses the bacterial envelope and then approaches its site of action (peptidoglycan of inner membrane). Thus, the binding of the fusion lysozyme to the bacterial membrane permeability barrier (LPS) appeared to be enhanced by the hydrophobic peptide at the C-terminus, resulting in disruption of the integrity of the outer membrane and subsequent perturbation of the inner membrane to inhibit the function of bacteria.

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