Bactericidal activity of human lysozymes carrying various lengths of polyproline chain at the C-terminus

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Abstract The amphiphilic polypeptide polyproline having different chain lengths was connected to the C-terminus of human lysozyme by the recombinant DNA technique. The hydrophobicity of human lysozyme increased with increasing length of the polyproline chain. Although the bactericidal activity of wild-type lysozyme is limited to Gram-positive bacteria and the hydrolytic activity of the mutant lysozyme decreased with increasing chain length of polyproline, the mutant lysozymes showed bactericidal activity to Gram-negative bacteria and the activity increased with increasing hydrophobicity of the mutant enzyme. Experiments with *Escherichia coli* phospholipid liposomes revealed that the mutant human lysozymes dissipated the valinomycin-induced transmembrane electrochemical potential, and the dissipation increased with increasing hydrophobicity. The increased hydrophobicity of the mutant enzyme may induce interaction of lysozyme with the outer membrane and subsequent penetration into the inner membrane of *E. coli*, resulting in an increase of bactericidal activity.

Key words: Human lysozyme; Hydrophobilization; Bactericidal activity; Mutation; Protein engineering

1. Introduction

The antibacterial activity of lysozyme is limited to Gram-positive bacteria. The enzyme catalyzes the hydrolysis of the β-(1,4)-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan in the bacterial cell wall [1], interacts with the lipopolysaccharide (LPS) layer in the outer membrane [2], and subsequently distorts the normal packing between phosphate groups of phospholipids and LPS by its polycationic properties [3]. The distortion results in perturbation of the structure of the outer membrane and stimulation of the susceptibility to peptidoglycan layer [4]. Despite its action on the outer membrane, lysozyme does not affect the viability of Gram-negative bacteria, while the same actions lead to lethality of Gram-positive bacteria.

However, Kato and coworkers reported that the covalent attachment of palmitic acid residue to the lysyl residue of hen egg-white lysozyme or the fusion of a hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro), which can form a β-strand conformation having the same length as the palmitic acid residue, to its C-terminus had bactericidal activity against *Escherichia coli* [5–7]. Nakamura et al. reported that the lysozyme-palmitic acid conjugate protected fish from infection with *E. tarda* [8]. They considered that the short hydrophobic chains promoted the penetration of the lysozyme molecule into the inner membranes of Gram-negative bacteria.

In this investigation, human lysozymes having different hydrophobicities were synthesized by gene-engineered attachment of polyproline chains at the carboxyl end of the enzyme and the bactericidal activities were investigated.

2. Materials and methods

2.1. Materials

The plasmid containing cDNA for human lysozyme [9] was kindly given by Takeda Chem. Ind. Ltd. (Osaka, Japan). Oligodeoxyribonucleotides used in the present investigation were synthesized by Sawady Technol. Co. (Tokyo, Japan). *Micrococcus luteus* cells were purchased from Sigma Co. (St. Louis, MO, USA).

2.2. Construction of expression plasmid of mutant human lysozyme

A primer 5′-GGT-GTT-GCC-CGG-GTC-GAC-CCG-GTC-GAC-3′ was used to introduce a *Sma*I endonuclease site instead of the stop codon of human lysozyme into pLY plasmid by site-directed mutagenesis.

Four primers, 5′-AAT-TCT-CAG-TCA-TCC-CGG-GGG-GCC-CAC-CAC-CAC-CAC-3′, 5′-CAC-CAC-CAC-CAC-CAC-CAT-AAT-AGG-TCG-ACC-GAC-CTG-TGC-TCA-3′, 5′-TGG-TGG-TGG-TGG-TGG-TGG-GCC-CCC-CGG-GAT-GAC-TGA-G-3′, and 5′-AGC-TTG-AGC-ACA-GGT-CGG-TCG-ACC-TAT-TAT-GGT-GGT-GGT-GG-3′, were annealed to form double strand DNA fragments encoding 10 residues of proline and the stop codon. Subsequently, the DNA fragments were annealed in the presence of pUC19 digested with *Eco*RI and *Hin*dIII and were ligated to construct pPR10 plasmid. The pPR10a plasmid encoding 10 residues of proline without the stop codon was constructed similarly. The pPR20 plasmid encoding 20 proline residues was constructed by insertion of the proline region into the pPR10a plasmid upstream of the pPR10 plasmid. The pPR30 plasmid was also constructed similarly. The construction of the plasmids was confirmed by electrophoresis and DNA sequencing.

pLY was digested with *Sma*I and *Sal*I endonucleases. The pPR10, pPR20 and pPR30 plasmids were digested with *Apa*I, a blunting kit (Takara, Japan) and finally *Sal*I. pLY-polyproline, which is the plasmid DNA encoding human lysozyme carrying polyproline, was constructed by ligation reaction. The construction of the plasmids was confirmed by electrophoretic analysis and DNA sequencing.

2.3. Expression of mutant human lysozymes

Lysozymes carrying various lengths of polyproline chain at the carboxyl end were expressed in *Saccharomyces cerevisiae* AH22 (MATa, *his3*, *leu2*) with pLY-pPR10, PLY-pPR20, or pLY-pPR30 plasmid. The human lysozymes carrying a polyproline chain consisting of 10, 20 or 30 residues at the carboxyl end were secreted because the plasmids contained a signal sequence [9].

2.4. Purification of mutant human lysozymes

The transformed yeast cells were precultivated in 100 ml of YPD (1% yeast extract, 1% peptone, 2% glucose) medium for 16 h and cultivated in 4 l of Burkholder medium for 4 days at 30°C. Cells in the late exponential phase were removed by centrifugation at 1700×*g* for 10 min and the medium having secreted proteins was concentrated to 400 ml by ultrafiltration using an AMICON model 2000 (Massa-

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chusetts, USA). The concentrate was loaded onto a CM-Toyopearl 650 C (Tosoh, Japan) column pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.5). After washing with 3 bed volumes of the same buffer, adsorbed protein was eluted in 50 mM sodium phosphate buffer (pH 6.5) containing 0.5 M NaCl and 10% glycerol. The active fractions were pooled and concentrated to 1 ml by ultrafiltration. Gel permeation chromatographic analysis was performed using a COSMOSIL 5Diol column (Nacalai Tesque, Kyoto, Japan) (20 mM phosphate buffer (pH 7.0)+0.1 M sodium sulfate at a rate of 1.0 ml/min).

2.5. Estimation of hydrophobicity

Hydrophobic chromatography was carried out using a TSKgel phenyl-5PW (Tosoh, Japan) column (linear gradient from 0.5 M phosphate-buffered solution (pH 7.0)+2.0 M ammonium sulfate solution to 0.5 M phosphate-buffered solution (pH 7.0) over 60 min at a rate of 1.0 ml/min).

2.6. Assay of hydrolytic activity

Hydrolytic activity was determined by hydrolysis of *p*-nitrophenyl tri-*N*-acetyl-β-chitotrioside. The substrate (6 mM) was dissolved in 0.5 ml of 40 mM citric acid buffer solution (pH 5.08) containing 10% of dioxane. The solution was incubated at 40°C for 3 min followed by addition of the lysozyme solution. The absorbance of *p*-nitrophenol released was monitored at 405 nm.

2.7. Assay of bactericidal activity

The suspension of *M. luteus* cell (1 mg/ml) was mixed with lysozyme in phosphate buffer (pH 6.2, $I=0.04$). The mixture was incubated at 25°C for 3 min. Ionic strength was adjusted with NaCl. The decrease of absorbance at 450 nm was measured. The activity of wild-type human lysozyme was taken as 100%.

The suspension of *E. coli* DH5α cells (10^5 cells/ml) was mixed with lysozyme (final concentration, 12 μg/ml) in 50 mM phosphate buffer solution (pH 7.0). The mixture was incubated at 37°C for 60 min and a diluted portion (50 μl) was pipetted out and plated onto LB agar plates. After incubation of the plates at 37°C overnight, the number of colonies was counted. The activity of wild-type human lysozyme was taken as 100%.

2.8. Measurements of membrane potential of E. coli phospholipid liposomes

Phospholipids were isolated from *E. coli* according to the method of Viitanen et al. [10]. The liposome was prepared by an extruder (Lipex Biomembrane Inc., Vancouver, Canada) through a series of polycarbonate membrane filters (Nucleopore Corp., Pleasanton, CA, USA). The potential was determined by measuring the fluorescence quenching of 3,3′-dipropylthiodicarbocyanine iodide [diS-C_3-(5)] using a Hitachi fluorescence spectrophotometer by the method reported by Kita et al. [11].

3. Results

3.1. Production of mutant human lysozymes

The purified lysozymes were analyzed by gel permeation chromatography. The mutant human lysozymes had a single peak in the elution profile. The analysis of amino acid composition showed that the mutant human lysozyme carrying 30 proline residues at the C-terminus had an alanine residue and 30 proline residues as designed in the recombinant gene. In addition, they were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The band positions of mutant human lysozymes were higher than expected. However, mass spectroscopy measurement demonstrated that the molecular weight of mutant human lysozyme carrying 30 proline residues was the same as the calculated mass (1.76×10^4 Da). It is known that hydrophobic proteins have unusual affinity for SDS because SDS-protein interaction forces are mainly hydrophobic [12,13]. Therefore SDS binding to the mutant human lysozyme should reduce the electrophoretic migration.

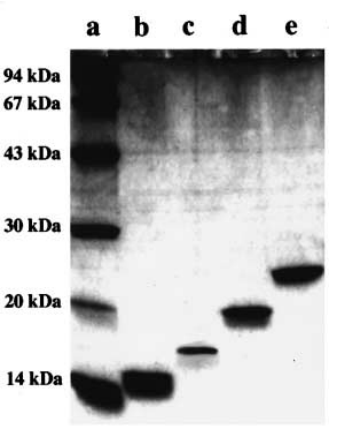

Fig. 1. Analysis of mutant human lysozymes by SDS-PAGE. Lane a: marker proteins; b: wild-type human lysozyme; c: mutant human lysozyme carrying 10 proline residues; d: mutant human lysozyme carrying 20 prolines residues; e: mutant human lysozyme carrying 30 proline residues.

3.2. Hydrophobicity of mutant human lysozymes

The hydrophobicity of mutant human lysozyme was investigated by hydrophobic chromatography (Fig. 2). The elution time was increased with increasing length of the proline chain and approached that of homo-polyproline.

3.3. Hydrolytic activity of mutant human lysozymes

Hydrolytic activity of mutant human lysozymes was reduced by connection of proline residues to the C-terminal (Table 1). Although K_m was not significantly affected by the mutation, k_{cat} decreased with increasing length of the polyproline tail. However, the mutant human lysozyme carrying 30 proline residues catalyzed hydrolysis of more hydrophobic (less water-soluble) substrate, penta-*N*-acetyl-β-chitopentaoside (21%) more than that of tri-*N*-acetyl-β-chitotrioside (11%), compared with wild-type human lysozyme. The hydrophobic mutant human lysozyme was considered to easily access the hydrophobic substrate.

Table 1
Hydrolysis of *p*-nitrophenyl tri-*N*-acetyl-β-chitotrioside (Tri) and *p*-nitrophenyl penta-*N*-acetyl-β-chitopentaoside (Penta), and bactericidal activity against Gram-positive and Gram-negative bacteria by various human lysozymes

Enzyme	Number of proline residues in the C-terminal	Tri			Penta	Bactericidal activity	
		Relative activity (%)	K_m (mM)	k_{cat} (min^{-1})	Relative activity (%)	Gram-positive (%)	Gram-negative (%)
Wild-type	0	100	2.27	2.31	100	100	100
Mutant	10	81	2.26	1.82	–	81	106
	20	37	2.27	0.77	–	38	122
	30	11	2.20	0.23	21	11	206

3.4. Bactericidal activity of mutant human lysozymes

Table 1 shows that although the bactericidal activity of mutant human lysozyme to Gram-positive bacteria was reduced with increasing length of the polyproline tail, the effect on Gram-negative bacteria (*E. coli*) was enhanced. The increase of hydrophobicity might increase the activity against Gram-negative bacteria.

Davies et al. [14] and Saint-Blancard et al. [15] reported that the bactericidal activity of wild-type lysozyme depended on the ionic strength. The activity of mutant lysozyme also depended on the ionic strength, although the optimum ionic strength of mutant human lysozyme was slightly different from that of wild-type human lysozyme (Fig. 3).

3.5. Interaction with liposome

The effect of mutant human lysozymes on the fluorescence quenching of diS-C_3-(5) by valinomycin in the *E. coli* phospholipid liposome was investigated. The addition of mutant human lysozymes to the liposomes hyperpolarized by valinomycin reversed the fluorescence quenching, but the addition of wild-type human lysozyme at the same concentration had no effect on the signal. The reversal of the fluorescence quenching by mutant human lysozymes may reflect their ability to disrupt the electrochemical potential. The increasing length of the polyproline tail increased the reversion and the significant collapse of the membrane potential in a way similar to the addition of nigericin. Taking into consideration that neither polyproline nor lysozyme affected the membrane potential, the combination of human lysozyme and hydrophobic polyproline is indispensable to affect the membrane potential.

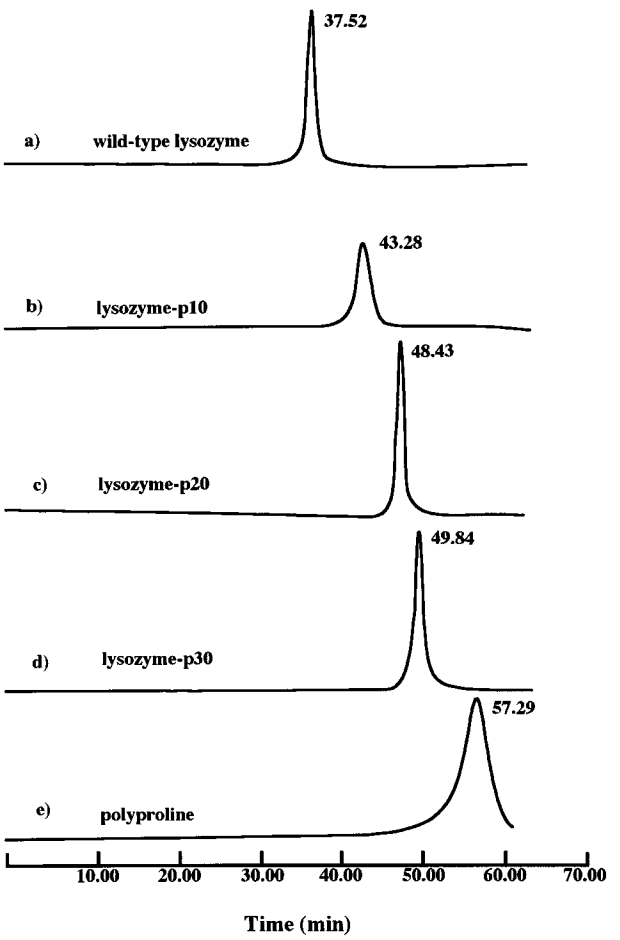

Fig. 2. Elution profile of mutant human lysozymes in the hydrophobic chromatography. a: Wild-type human lysozyme; b: mutant human lysozyme carrying 10 proline residues (lysozyme-p10); c: mutant human lysozyme carrying 20 proline residues (lysozyme-p20); d: mutant human lysozyme carrying 30 proline residues (lysozyme-p30); e: polyproline (8 kDa).

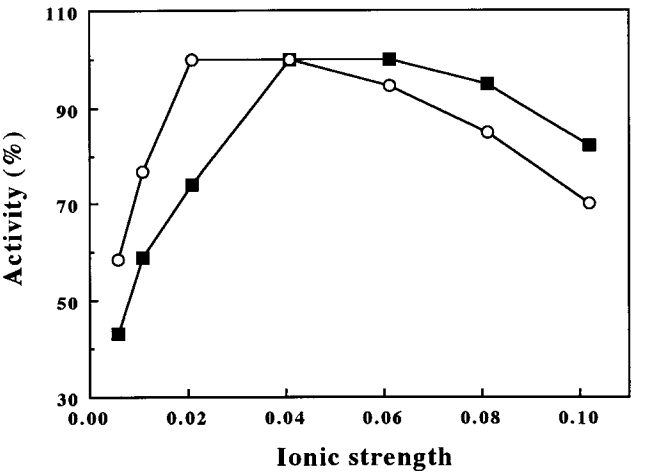

Fig. 3. Influence of ionic strength on the lysis of *M. luteus* cells by wild-type (■) and mutant human lysozymes carrying 30 proline residues (○) at pH 6.2. Maximal activity of each human lysozyme is represented as 100%.

4. Discussion

Various types of antimicrobial peptides, including post-translationally modified peptides, have been found and these peptides have characteristic secondary structure or amino acid composition [16–18]. A group of peptides, including cecropins, magainins, and mellithitin, take an α-helical structure in lipid membrane. The α-helical structure has been found in many other antimicrobial peptides [19,20]. On the other hand, peptides having an antiparallel β-sheet structure are known [21]. Some peptides contain a high percentage of specific amino acids such as the proline- or arginine-rich bovine peptides, Bac 5 and Bac 7 [22], the porcine peptide PR-39 [23], and the tryptophan- or proline-rich peptide indolicidin [24,25], tryptophan-rich peptide [26]. Recently Falla et al. [24] reported that indolicin has a weak poly(L-proline)-II-type extended helix structure. Skelavaj et al. [27] reported that two novel cathelicidin-derived peptides having a cationic N-terminal region and an amphipathic α-helical conformation followed by a hydrophobic C-terminal tail had antimicrobial activity.

In the present investigation, the proline residues may form a polyproline helix structure to result in increased hydrophobicity. Considering that the pK_a of human lysozyme is 11 [28], the protein is positively charged under neutral conditions. A perturbation of membrane potential by the mutant human lysozymes should occur either by the hydrophobic interaction of the polyproline tail with lipid membrane or by electrostatic interaction between the positively charged groups of the lysozyme molecule and the anionic polar heads of the phospholipids of the membrane. The physico-chemical property rather than the hydrolytic activity of the mutant lysozymes should have a greater contribution to the bactericidal activity, taking

into account that the mutant human lysozymes had very low activity. The ability of mutant lysozymes to damage the cell membrane may account for their bactericidal action to *E. coli*.

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