Antimicrobial Effects of Lysozyme against Gram-Negative Bacteria Due to Covalent Binding of Palmitic Acid

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Chicken lysozyme was chemically modified to various degrees with the *N*-hydroxysuccinimide ester of palmitic acid. Lytic activity of lysozyme against *Micrococcus lysodeikticus* was slightly decreased with the increase of attached palmitoyl residues up to two residues per molecule, whereas further modification resulted in unfavorable insolubility and less active lysozyme. Lysozyme derivatives exhibited a substantial antimicrobial activity against Gram-negative bacteria (*Escherichia coli* wild type 3301) without addition of EDTA or heating. The most potent derivative was the lysozyme incorporating two palmitoyl residues (P2-LZ). Lytic activity of lysozyme derivatives in the presence of purified LPS (outer membrane lipopolysaccharides of *E. coli*) against *M. lysodeikticus* was consistently decreased with an increase in the degree of modification with palmitic acid. This suggested that enhanced activity of palmitoyl lysozyme molecules occurred via membrane insertions through the LPS zone. The foam stability and emulsifying activity of lysozyme were markedly promoted in response to the increase in the extent of palmitoylation up to four residues per molecule. Thus, this approach indicates that palmitoyl lysozyme in the formulated food systems would provide a novel class of therapeutic agents and food safety.

INTRODUCTION

Lysozyme is an enzyme (129 amino acid residues) that catalyzes the hydrolysis of the β-1,4-glycosidic linkage of the peptidoglycan in the bacterial cell wall and which has been used as anti-inflammatory drug (Imoto et al., 1972). However, an important prerequisite for access in the application of a pharmacologically active protein molecule is broadening its specificity. Unfortunately, antibacterial action of lysozyme is limited to Gram-positive strains; it does not act on Gram-negative bacteria, including food-borne pathogens. Thus, any modification of the properties of lysozyme that could render it useful on both Gram-negative and Gram-positive bacteria would be an important contribution. Unlike Gram-positive, the bacterial lipopolysaccharide (LPS) layer along with proteins and phospholipids is the major component on the outer surface of Gram-negative bacteria. Access of lysozyme to the peptidoglycan layer of the cell wall is hindered by the outer lipopolysaccharide layer. This explains the resistance of Gram-negative strains to the lytic action of lysozyme.

There are at least two options open to us in achieving optimal food functionality and diverse lytic activity of hen egg white lysozyme. In the conventional one, efforts have been directed toward the creation of a surfactant molecule by coupling dextran to lysozyme (Nakamura et al., 1990). The lysozyme–dextran conjugate had a desirable antimicrobial activity against Gram-negative bacteria but only when the cells were heated at 50 °C for 20 min with conjugate. Here, our strategy of designing an effective anti Gram-negative agent is based on the attachment of a hydrophobic chain to lysozyme to enable it to fuse into the outer membrane, an amphitropic protein mimetic approach. Amphitropic proteins are lipid-binding proteins, such as α-actinin and vinculin, that can traverse through the cell membranes reversibly (Burn, 1988). This rational approach could provide a simple functional molecule, not necessarily target-specific, which could access the outer membrane LPS layer by covalently linking the active molecule (lysozyme) to a hydrophobic ligand (palmitic acid) to facilitate its delivery to the site of action (peptidoglycan of cytoplasmic membrane) to perform its task. This paper reports that a lysozyme derivative incorporating two palmitoyl residues is very potent against Gram-negative bacteria (*Escherichia coli*) due to its improved surface activity.

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MATERIALS AND METHODS

Lysozyme crystallized five times was provided by Q. P. Corp., Tokyo. The microbial substrate of lysozyme, *Micrococcus lysodeikticus* cells, was purchased from Sigma Chemical Co. (St. Louis, MO). LPS was prepared from *Escherichia coli* WT-3301 by extraction according to a modified phenol–water procedure as reported earlier (Morrison and Leive, 1975). Unless otherwise stated, all reagents used in this study were of reagent grade.

Modification of Lysozyme. Palmitic acid was esterified with *N*-hydroxysuccinimide as reported previously (Haque and Kito, 1982). The palmitic acid was covalently attached to a lysyl residue of lysozyme by base-catalyzed ester exchange according to the method of Haque et al. (1982), with minor modifications as follows: The degree of incorporation was controlled by adjusting the molar ratio of *N*-hydroxysuccinimide ester of palmitic acid to lysozyme. The mole content of lysine in lysozyme was considered to be seven residues (six side chains of lysine plus the amino terminus). Thus, the preparations were performed at a mole ratio of the *N*-hydroxysuccinimide ester of palmitic acid to lysozyme of 1, 2, 3, or 4 to obtain lysozyme derivatives P1-LZ, P2-LZ, P3-LZ, and P4-LZ, respectively. Ten milliliters of tetrahydrofuran (THF) containing the appropriate amount of *N*-hydroxysuccinimide ester of palmitic acid (0.7, 1.4, 2.1, and 2.8 mM final concentration in the reaction mixture) was gradually added to 40 mL of 50 mM potassium phosphate buffer, pH 7.4, containing a single concentration of lysozyme (0.7 mM). While the mixture agitated at 25 °C, the pH was brought to 9.0 with NaOH (1 N). The reaction mixture was incubated for 1 h with gentle agitation. The pH was then adjusted to 7.0 with HCl (1 N), and the mixture was subsequently dialyzed for 2 days at 4 °C against buffer containing 1 M NaCl. Thereafter, the reaction was washed five times with ice-cold diethyl ether and alternately centrifuged at 2000 rpm for 5 min at 0 °C. The protein portion was then dialyzed exhaustively for 3 days against three daily exchanges of distilled water. The lysozyme derivatives (P1-LZ, P2-LZ, P3-LZ, and P4-LZ) were then lyophilized and used for the following tests. A control sample was taken through all steps of preparation without addition of the *N*-hydroxysuccinimide ester of palmitic acid in THF.

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Protein concentration of the lysozyme derivatives was assessed using the modified Lowry method of Miller (1959).

Protein solubility was determined by measuring protein concentration after the solution of lysozyme derivative (0.1% w/v protein in 50 mM potassium phosphate buffer, pH 7.4) was passed through a membrane filter (0.45 μm) according to the Miller (1959) method. Solubility was expressed as percent of protein concentration in the filtrate of lysozyme derivatives with respect to that of control sample.

The degree of modification was determined by measuring the free amino groups of control and modified lysozyme, using TNBS reagent, as reported earlier (Kato et al., 1989).

Antimicrobial Action. The lytic action of lysozyme derivatives against *M. lysodeikticus* cells was determined according to turbidometric methods (Muraki et al., 1988) based on the decrease in turbidity of a cell suspension following the addition of lysozyme derivatives. The lysis of cells in 50 mM potassium phosphate buffer (pH 8.0) was monitored at 600 nm. A 100-μL portion of the lysozyme derivative solution (final concentration, 0.84 μg/mL) was added into 1.9 mL of *M. lysodeikticus* cell suspension (final concentration, 250 μg/mL dry weight in the same buffer). This lysis mixture gave initial OD of 0.8–0.85 at the onset of the experiment. The decrease in absorbance at 600 nm (25 °C) was monitored using a Hitachi U-2000 recording spectrophotometer.

Lytic action of lysozyme derivatives 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 the lysozyme derivatives (final concentration, 0.84 μg/mL). The mixtures were incubated at 37 °C for 15 min before tempering and mixing with the *M. lysodeikticus* cell suspension (250 μg/mL, OD of 0.85). The final volume of the lysis mixture was always kept 2 mL.

To measure the antimicrobial action of lysozyme derivatives against *E. coli* (wild type 3301), a 4.5-mL *E. coli* cell suspension (10^6 cells/mL in 10 mM sodium phosphate buffer, pH 7.0) was mixed with a 0.5-mL solution of the lysozyme derivatives (final concentration, 0.05% w/v). In a separate set of experiments, ethylenediaminetetraacetic acid (EDTA) was added to the buffer (final concentration, 5 mM). The mixture was kept at room temperature for 15 min. A 100-μL portion was plated out onto MacConkey agar. Colonies were counted after incubation at 35 °C for 24 and 48 h.

Functional Properties of Modified Lysozyme. Foaming properties of palmitoyl lysozyme was determined by measuring the conductivity of foams produced when air at a constant flow rate of 90 cm^2/min was introduced for 15 s into 5 mL of 0.5% protein concentration in 0.1 M phosphate buffer, pH 7.4, in a vertical glass column (2.4 × 30 cm) with a glass filter at the bottom (Kato et al., 1983). The conductivity of foams was measured by an electrode that had a cell. The cell was fixed inside the glass column 1 cm apart and 2.4 cm above the filter and was connected to a conductivity meter (Kyoto Electrics Industry Co., Model CM-07). Foaming power was defined as the maximum conductivity of foams produced after 15 s when air was introduced. Foam stability was calculated from conductivity curves as disappearance time of foam.

Emulsifying properties of palmitoyl lysozyme were determined according to the method of Pearce and Kinsella (1978). An emulsion was prepared by homogenizing 1.0 mL of corn oil and 3.0 mL of a 0.1% control or modified lysozyme in 50 mM potassium phosphate buffer, pH 7.4, using an Ultra Turrax machine (Hansen & Co.) at 12 000 rpm for 1 min at 20 °C. Fifty microliters of the formed emulsion was taken from the bottom of the container after the desired time and diluted with 5 mL of a 0.1% SDS solution. The absorbance of the diluted emulsions was then measured at 500 nm.

All tests in this study were performed in triplicate through two independent experiments except the antimicrobial activities against *E. coli*, which were carried out only in duplicate.

RESULTS

The optimal number of palmitoyl residues incorporated to lysozyme was preliminarily examined in the range of one to six residues per molecule. Incorporation of more than four palmitoyl residues to a lysozyme molecule led to substantial loss in solubility and unfavorable decrease in enzymatic activity (data not shown). For this reason, the maximum degree of modification adopted in this study was limited to four residues of palmitic acid per molecule of lysozyme, where appreciable solubility and activity could be preserved. Since the attachment method of palmitic acid used in this study is specific to ε-amino groups of lysyl residues, we speculated that measuring the free amino groups of lysozyme derivatives and comparing it with that of control sample would give a rational indication to the degree of incorporation. Figure 1 shows the changes in the free amino groups of lysozyme modified to varying extents with palmitic acid. The data were expressed as the ratios to value of unmodified lysozyme (control). Since the mole content of free amino groups in lysozyme has been considered to be seven, a minimum estimate of stoichiometry of palmitoylation was made on the basis of the result in Figure 1. Thus, the stoichiometry of palmitoylation of lysozyme derivatives P1-LZ, P2-LZ, P3-LZ and P4-LZ is 0.7, 1.4, 1.9, and 3.2 mol of palmitic acid/mol of lysozyme, respectively. It was proven that the decrease in the free amino groups of lysozyme is in good agreement with the anticipated degree of modification adjusted by controlling the mole ratio of *N*-hydroxysuccinimide ester of palmitic acid to lysozyme molecules. The relationships between the number of attached palmitoyl residues to lysozyme and solubility or turbidity are shown in Figure 2. There was a gradual decrease in protein solubility with concomitant increase in solution turbidity with an increase in the degree of palmitoylation. When four palmitoyl

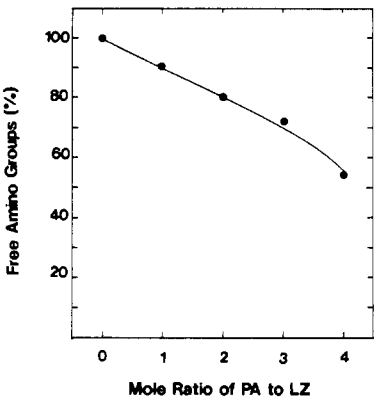

Figure 1. Changes in free amino groups of lysozyme as a function of increasing molar ratio of *N*-hydroxysuccinimide ester of palmitic acid in the reaction mixture. PA, palmitic acid; LZ, lysozyme.

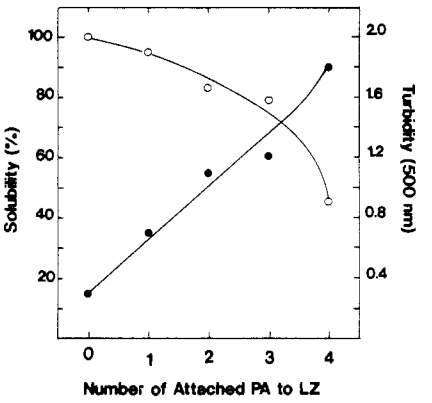

Figure 2. Protein solubility and turbidity of modified lysozyme to various degrees with palmitic acid. Values of solubility of modified samples are represented as the ratio to nonmodified control: (○) solubility; (●) solution turbidity at 500 nm.

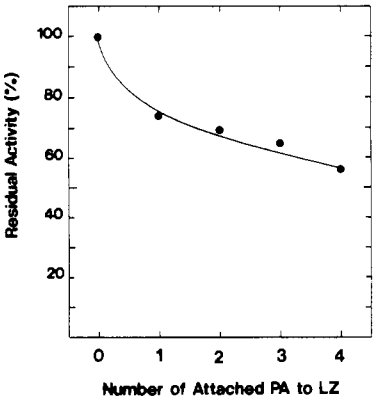

Figure 3. Lytic activity of lysozyme modified to various degrees with palmitic acid. (0) Control lysozyme (as 100% activity).

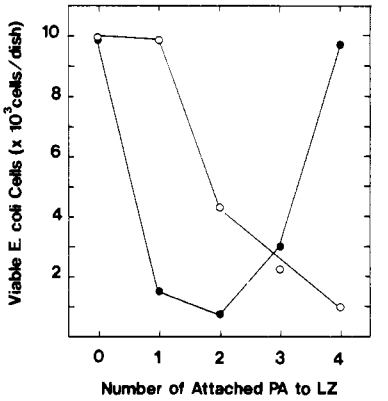

Figure 4. Antimicrobial activity of lysozyme modified to various degrees with palmitic acid against *E. coli* assessed in the presence (O) or absence (●) of EDTA.

residues were covalently attached to lysyl residues of lysozyme, 45% of protein solubility was restored. Further increases in the degree of modification resulted in complete loss of solubility (data not shown). It should be noted that the solubilities of lysozyme derivatives P1-LZ and P2-LZ (bearing one and two palmitoyl residues, respectively), were 96% and 83%, respectively. This suggests that attachment of one or two palmitoyl residues to lysozyme is the most appreciable degree of modification in terms of conserving a considerable degree of solvation.

Lytic activities of lysozyme modified to various extents are shown in Figure 3. The percent of residual activity of lysozyme derivatives is represented as the ratios to value of control. There was a remarkable decrease in the residual activity with the increase of the degree of modification up to four residues of palmitic acid. However, the dramatic loss in activity was observed when four residues of palmitic acid were attached (P4-LZ), where the residual activity became 56%. On the other hand, modification of lysozyme with palmitic acid up to three residues per molecule had no undesirable decrease in its enzymatic activity. In other words, more than 70% of lytic activity of lysozyme was conserved with modification up to three residues. Since palmitic acid was covalently attached to ϵ-amino group of lysine residue of lysozyme (total six residues), the net positive charge of lysozyme molecule would decrease by 1 unit with each attached palmitoyl residue. It has been reported that the net positive charge of lysozyme plays an important role in the initiation and efficiency of its lytic action against the negatively charged cell wall of *M. lysodeikticus* (Davies et al., 1969; Imoto et al., 1974; Muraki et al., 1988). The effect of surface charge modification on the enzymatic activity was also explored using chicken lysozyme and was found to play a critical role in the enzymatic behavior (Frieden, 1956; Yamasaki et al., 1968a,b; Davies and Neuberger, 1969). In the current study the decreased residual activity of palmitoyl lysozyme is in full agreement with the above-mentioned results because the surface positive charge has been partially abolished by modification. However, a reasonable enzymatic activity was restored when the number of attached palmitoyl residues did not exceed three residues per molecule of lysozyme.

Having identified the optimum degree of modification with which appreciable solubility and lytic activity could be conserved, we systematically explored the antibacterial activity of modified lysozyme against *E. coli*. Figure 4 shows the activity of lysozyme on *E. coli* as a function of the degree of modification with hydrophobic ligand (palmitic acid). As shown, the most potent lysozyme type was to that modified with two palmitoyl residues (P2-LZ). Moreover, the modified lysozyme with a single residue was also potentially lethal for *E. coli*. It should be pointed out that incorporation of four palmitoyl residues to lysozyme (P4-LZ) resulted in complete suppression of the observed antibacterial activity against *E. coli* observed with the lower degree of modification, even though it maintained 56% of its lytic activity against the Gram-positive *Micrococcus*. Presumably, the increased number of hydrophobic chains on the surface of the lysozyme molecule tightened the interaction of lysozyme with the outer membrane of *E. coli* cells through strong hydrophobic forces; this may have embedded the active site of molecule in membrane and subsequently eliminated its activity against the peptidoglycan. The activity of lysozyme derivatives on *E. coli* in the presence of 5 mM EDTA are also given in Figure 4. P1-LZ derivative has no detectable effect on *E. coli* viability, while further incorporation of palmitic acid resulted in a constant decrease in the viability of *E. coli* up to four palmitoyl residues per molecule (P4-LZ). It can be noted that the number of viable cells in the presence of EDTA and P4-LZ was approximately identical to that of P2-LZ in the absence of EDTA. This suggests that the lethal effect of palmitoyl lysozyme is more prominent in the absence of EDTA rather than in its presence, in spite of the well-known killing effect of EDTA on Gram-negative bacteria (spheroplasting). The retardation of antibacterial potency of palmitoyl lysozyme caused by EDTA remains unclear, and elucidation of this phenomenon by experimental data is now in progress.

To investigate the role of palmitoyl residues attached to lysozyme on the anticipated penetration or fusion of palmitoyl lysozyme into the outer membrane LPS of *E. coli*, we purified lipopolysaccharides (LPS) of *E. coli* and examined the binding affinities to lysozyme derivatives by monitoring the residual lytic activity of lysozyme on *M. lysodeikticus* after treatment with the LPS components. Figure 5 shows the lytic activity of lysozyme modified to various degrees with palmitic acid in the presence of different concentrations of purified LPS. It is shown that LPS progressively decreases the residual activity of control lysozyme (unmodified) in a dose-dependent manner up to 30-fold excess of LPS concentration. On the other hand, when lysozyme was modified with one palmitoyl residue (P1-LZ), the inhibition potency of LPS to lysozyme activity was significantly promoted as the curve was displaced to lower activity at any LPS concentration. Further increases in the number of palmitoyl residues attached to lysozyme had an additional displacement of the curves to lower activities up to four palmitoyl residues per molecule. In phenomenological terms, the decreased enzymatic activity of lysozyme in response to LPS is related to the formation of tightly bound lysozyme–

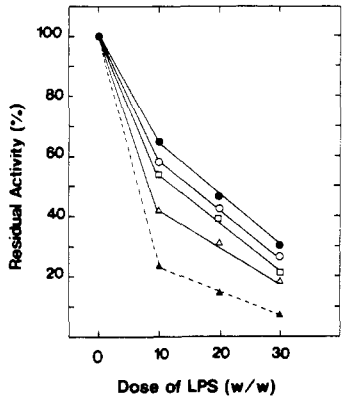

Figure 5. Effect of LPS on the lytic activity of lysozyme as a function of degree of modification with palmitic acid: (●) control; (○) P1-LZ; (□) P2-LZ; (△) P3-LZ; (▲) P4-LZ.

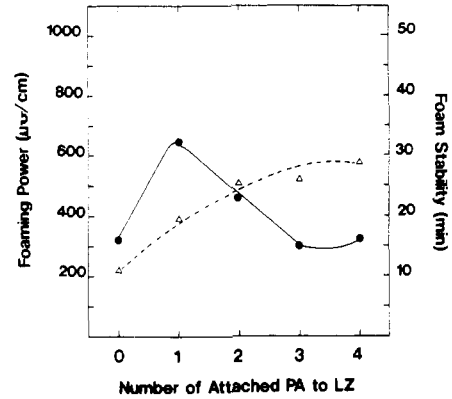

Figure 6. Relationship between foaming properties and degree of modification of lysozyme with palmitic acid: (●) foaming power; (△) foam stability.

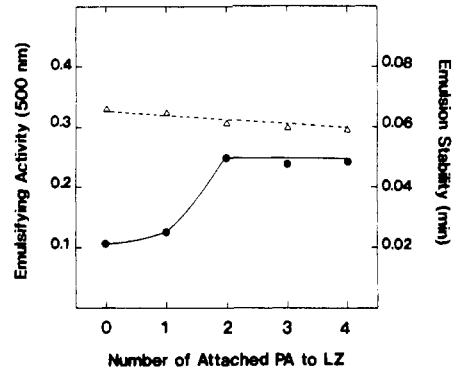

Figure 7. Relationship between emulsifying activity and degree of modification of lysozyme with palmitic acid: (●) emulsifying activity; (△) emulsion stability.

LPS complex and the fact that the interaction is primarily dictated by hydrophobic interactions (Ohno and Morrison, 1989a,b). Taking all results together, the data demonstrate that the binding of lysozyme to bacterial outer membrane LPS could be substantiated by coupling hydrocarbon chain to lysozyme. Therefore, our rational design of an antimicrobial compound to Gram-negative bacteria from lysozyme through enhancement of its surface hydrophobic moment is confirmed. Modified lysozyme with four palmitoyl residues (P4-LZ) exhibited the highest affinity to LPS (Figure 5), while it had no effect on the growth of *E. coli* (Figure 4). It is probable, however, that attachment of four residues of fatty acid to lysozyme may have rendered the protein less soluble, having high tendency to form micelle which in turn led to significant decrease in its enzymatic activity. This was confirmed from the results shown in Figures 2 and 3. In addition, the results of Figure 5 suggest that the active molecule (lysozyme) in P4-LZ appears to be tightly immobilized in the outer LPS membrane, where its antimicrobial activity was diminished. However, the data suggest that the optimal inhibitory potency to Gram-negative bacteria would be favorably gained with moderate palmitoylation. One or two residues per molecule would sustain the appropriate functional enzymatic activity upon penetration through the bacterial envelope and reaching the site of action (peptidoglycan).

A protein, even though it may have excellent biological activity, will have no impact on human nutrition unless it has functional properties suitable for incorporation into food systems. Foaming and emulsifying properties of a protein are the most important necessities to guarantee its involvement in formulated food systems. Furthermore, since the amphiphilic nature of lysozyme was altered by such modification, it is logical to investigate the effect of palmitoylation on the major surface functional properties of lysozyme. The relationship between foaming properties and degree of modification is shown in Figure 6. Foam stability was progressively increased with the increase in the degree of palmitoylation of lysozyme. In contrast, foaming power was improved 2 times with incorporation of one palmitoyl residue, where it began to decrease with further increase in the number of attached palmitoyl residues. However, there was no deterioration in the foaming power of palmitoyl lysozyme compared with control lysozyme at any degree of modification adopted in this study. Thus, the results obtained from palmitoyl lysozyme with one and two residues are very promising, because both parameters of foamability were promoted by this degree of modification; this is especially noted since lysozyme has very poor foaming and emulsifying properties. Figure 7 shows the relationship between emulsifying properties and degree of palmitoylation of lysozyme. There was a substantial improvement in the emulsifying activity of lysozyme proportional to the increase in the number of attached palmitoyl residues, up to two residues per molecule of lysozyme, where it became constant. In contrast to the positive influence of modification on the emulsifying activity of lysozyme, the emulsion stability was adversely affected. This suggests that enhancement of the surface hydrophobicity of poor emulsifier proteins, such as lysozyme, could significantly improve their emulsifying capacity but not their emulsion stability. Moreover, this indicates that hydrophobicity is not the sole determinant for the emulsion stability of a protein. It seems very likely, therefore, that many other molecular elements other than hydrophobicity are involved such as protein flexibility (Kato et al., 1985), surface charge (Adams et al., 1971), conformational stability (Kato et al., 1990; Song and Damodaran, 1987), solubility (Halling, 1981; Chobert et al., 1988), and molecular size (Kato, 1990).

DISCUSSION

Our strategy of designing lysozyme for antimicrobial activity against Gram-negative bacteria was based on coupling a aliphatic hydrocarbon carrier to lysyl residues so as to facilitate its fusion and penetration through the bacterial protective barrier (outer membrane). Lysozyme elaborated in this study is an amphipathic molecule when it was moderately modified with palmitoyl residues. The positively charged lysozyme molecule constitutes the polar head, and the hydrocarbon chain (palmitic acid) is the nonpolar tail. These are characteristics of the so-called polar lipids, the most abundant membrane lipids of

bacterial cells. Of the many different types of proteins found capable to pass through outer membrane of *E. coli*, for example, all appear to possess a hydrophobic sequence (e.g., hemolysin) or contain a covalently bound fatty acid (amphitropic proteins such as α-actinin and vinculin). Therefore, it is rational to expect the results obtained in this study. The data confirm that lipophilization of lysozyme has rendered the molecule potent to penetrate the protective barrier of *E. coli*, as model for Gram-negative bacteria, since lysozyme was inaccessible to its substrate (*M. lysodeikticus*) in the presence of LPS, as shown in Figure 5. In parallel, modified lysozyme thus was proved to be a very potent inhibitor to *E. coli* growth without further treatment. This suggests that palmitoyl lysozyme may be associated with zones of LPS of cell envelope through hydrophobic interaction. As a consequence, lysozyme molecules may pass as they traverse the bacterial envelope and then approach its site of action (peptidoglycan of inner membrane), through lateral or basal movements of polar heads between the two planes of bilayer of outer membrane (flip-flop). This proposal is dictated from the dramatic decrease in the number of viable *E. coli* cells when treated with modified lysozyme to a moderate extent. The major difficulties in addressing this mechanism are that the palmitoyl lysozyme may fuse into the outer membrane and subsequently internalize into the periplasm so transiently that it is impossible to observe using currently available techniques. However, the development of turbidity (Figure 2) with modification clearly indicates the formation of micelles of palmitoyl lysozyme. This is very consistent with the decrease in solubility assessed by passing the solution of lysozyme derivatives through a membrane filter. It is well documented that the micelle of polar lipid (liposome) can be fused and integrated into bilayer lipids of cell membrane under some conditions (Lehninger, 1987). It appears, therefore, that when the micelles of palmitoyl lysozyme were fused into the cell envelope, the positively charged lysozyme molecules (polar head) have come in contact with or approached the negatively charged phospholipid bilayer of inner membrane, most probably via zones of adhesion between the outer and inner membranes. As a result there may be an initial electrostatic interaction between the positively charged groups of moderately modified lysozyme and negatively charged head groups of phospholipids, thus leading to localization of lysozyme in the vicinity of the site of action.

The relative propensity of a protein to penetrate the lipid bilayer may be imposed by its hydrophobicity, the length of the hydrophobic moment, and the degree of exposure to the aqueous phase. The results of this study revealed that modified lysozymes with one or two palmitoyl residues are the more potent antibacterial compounds against *E. coli*, suggesting that lysozyme with one or two palmitoyl residues can easily form amphipathic molecule which may access the outer membrane without strengthened insertion into lipid bilayer and reaching the site of action. On the other hand, increasing the number of attached palmitoyl residues may strengthen the insertion of lysozyme into the envelope or even the formed micelle itself and, subsequently, hinder the exposure of the active site to the inner surface of membrane where asymmetric hydrophobic interactions prevail. Moreover, the more palmitic acid attached to lysyl residues of lysozyme the less the net positive charge of the molecule. Interestingly, the antibacterial potency against *E. coli* of compound P1-LZ was abolished and that of P2-LZ and P3-LZ was reduced, while the potency of P4-LZ was very high in the presence of EDTA. It is very probable that the reduction of antibacterial activity by EDTA contributed to the elimination of cations, such as Mg^{2+}, from the negatively charged polar heads of lipid bilayer of outer membrane and hence the increase of the electrostatic potential between positively charged groups (lysozyme modified with a moderate number of fatty acid) and the negatively charged exposed heads of the envelope. As a consequence, lysozyme might be entrapped into the outer membrane. In contrast, the highly modified lysozymes (P3-LZ and P4-LZ) were more potent inhibitors for *E. coli* as their net positive charges were less, and the access of considerable residual lysozyme activity (56%) to the plasma membrane would be more dominant (spheroplasting). It is also probable that the highly modified lysozyme P4-LZ had very strong hydrophobic interaction with the outer (LPS) membrane. Consequently, this modified lysozyme type can hardly reach the peptidoglycan. With EDTA treatment (disruption of the integrity of the outer membrane), one would expect that the antimicrobial action of this highly modified lysozyme type should be accentuated because it is not being excluded from the peptidoglycan in cells when the outer membrane integrity has comprised EDTA. This postulation is the most conceivable since the data in Figure 5 show that EDTA does promote the antimicrobial effects of highly modified lysozyme types (P3-LZ and P4-LZ). However, elucidation of this phenomenon is now in progress using labeling with photoreactive and isotope reagents to monitor the behavior of modified lysozyme to varying degrees.

Proteins at liquid interfaces play important roles in many biological systems and industrial product formulations. Thus, we have already verified the improvement of surface properties of lysozyme due to modification with palmitic acid. The results revealed that foam stability and emulsifying activity of lysozyme were greatly promoted in proportion to the increase in the degree of modification. These two functional properties of a protein are known to be largely influenced by the amphiphilic balance of a protein molecule. As a result of the attachment of a fatty acid chain to lysozyme, the hydrophobic interactions among palmitoyl lysozyme molecules at the interface may have enhanced and hence stabilized the formed air/water film by retarding the rate of liquid drainage. It was observed that the moderately palmitoylated lysozyme (P1-LZ and P2-LZ) exhibited substantial improvement in foaming power, while those having three and four palmitoyl residues were similar to control. This indicates that the optimum foamability as well as antibacterial activity of lysozyme could be obtained by attaching the minimum number of palmitoyl residues. Emulsifying activity of lysozyme was also increased to favorable extent through modification as the amphiphilic nature is balanced. In contrast, emulsion stability was slightly decreased as a function of the increase in the degree of modification, suggesting that the ion–dipole interaction of water with the polar groups of lysozyme molecules has been decreased and might decrease emulsion stability by facilitating the coalescence of oil droplets after the creation of interface.

This approach heralded fascinating opportunities for engineering potentially active proteins, such as lysozyme, that are lethal to Gram-negative and Gram-positive bacteria. In addition, they possess a remarkable improvement in surface properties necessary for incorporation into formulated food or drug systems. However, further study is needed to elucidate the mechanism underlying the behavior of lysozyme following modification with different fatty acids against pathogenic bacteria that play an

important role in human, veterinary, and plant diseases as well as enzymes that have a potential commercial value.

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